Insecticide resistance in Drosophila melanogaster and Ctenocephalides felis

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Insecticide resistance in *Drosophila melanogaster* and *Ctenocephalides felis*

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Abstract

Insecticide resistance represents a major challenge for agricultural production and human health. Two main mechanisms for resistance have been identified; target-site and metabolism based resistance. This thesis examines an example of target-site based resistance in the ectoparasite, *Ctenocephalides felis* and an example of metabolism-based resistance in the model organism *Drosophila melanogaster*.

The use of *D. melanogaster* in insecticide resistance research has facilitated the study of resistance mechanisms despite not being a pest insect. The over expression of the Cytochrome P450 monoxygenase, *Cyp6g1* is associated with DDT resistance in *D. melanogaster*. The insertion of an *Accord* transposable element in the 5' of *Cyp6g1* was previously shown to be necessary and sufficient for *Cyp6g1* over expression.

In this thesis *Cyp6g1* expression is shown to be tissue specific. Data indicate *Cyp6g1* is involved in the metabolism of DDT and the artificial substrate methoxy resorufin ether. The *Accord* mutation has reached fixation in populations outside Africa. This may be due to cross-resistance to other compounds or little or no cost to *Cyp6g1* over expression. Life history analyses and population cage studies indicate that the resistant individuals show an increased fitness in the laboratory. The role of *Cyp6g1* in reproduction is examined and the over expression of *Cyp6g1* in sperm is identified.

Finally the cat flea, *C. felis*, is an important ectoparasite of domestic animals. Resistance to cyclodiene insecticides and fipronil has been associated with a single point mutation within the RDL subunit of the GABA receptors. This point mutation is highly conserved within a number of insect species and has been identified in *C. felis*. A TaqMan based diagnostic has been developed and applied to flea populations in an attempt to identify the resistance allele in populations and show a correlation between the putative resistance mutation and reduced sensitivity to fipronil.

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Abbreviations

µl	microlitre
20E	20-hydroxyecdysone
AChE	Acetylcholinesterase
Acp	Accesory gland protein
ANOVA	Analysis of Variance
BCIP	5'Bromo-4-chloro-3-indolyl phosphate
BIDN	3, 3-bis-trifluromethyl-bicyclo-[2,2,1]heptane-2,2-dicarbonitrile
bp	Base pair
BSA	Bovine serum albumin
Bt	Bacillus thuringiensis
CA	Corpora allata
CI	Cytoplasmic incompatibility
CNS	Central Nervous System
ddH2O	Double distilled water
DDT	1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
DDT	Dichlorodiphenyltrichloroethane
DDT-R	DDT resistant
DDT-S	DDT susceptible
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
FAD	Feline allergic dermatitis
g	gram
GABA	γ-aminobutyric acid
GABA-R	GABA receptor
GFP	Green fluorescent protein
GluCl	Glutamate-gated chloride channel
GST	Glutathione-S-transferase
h	hour
HRP	Horseradish peroxidase
IRAC	Insecticide resistance action committee

JH	Juvenile hormone
JHA	Juvenile hormone analogue
JHB3	JH methyl 6,7;10,11-bisepoxyfarnesoate
kdr	Knockdown resistance
l	litre
LC50	Lethal concentration 50
Lcch3	Ligand-gated chloride channel homologue 3
LC-MS	Liquid chromatography-mass spectrometry
LTR	Long terminal repeat
M	Molar
MBP	Maltose binding protein
MCRS	Male competitive reproductive success
min	minute
ml	millilitre
MROD	Methoxy resorufin ether
MWCO	Molecular weight cut-off
nAChR	Nicotinic acetylcholine receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium chloride
NHS	N-Hydroxysuccinimide
O/N	Overnight
OP	Organophosphate
PAGE	Polyacrylamide gel electrophoresis
PASA	PCR base amplification of specific alleles
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonylfluoride
POPs	Persistent organic pollutants
ppb	Parts per billion
PTX	Picrotoxin
Rdl	Resistance to dieldrin
REN	Restriction endonuclease
RNA	Ribose nucleic acid
RNAi	RNA interference
Rpm	Revolutions per minute

s	second
SDS	Sodium dodecyl sulphate
SE	Standard Error
SP	Sex peptide
spp.	Species
SSCP	Single stranded conformational polymorphism
TE	Transposable element
UAS	Upstream activation sequence
VSSC	Voltage-sensitive sodium channel
WHO	World Health Organisation

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CHAPTER 1

Introduction

1.1 DROSOPHILA SPP.

1.1.1 Life history and ecology

Drosophila spp. are found worldwide, with relatively more species in the tropical regions. They are found in a diverse array of habitats including desert, tropical rainforest, swamp regions, alpine zones and urban areas. Most species breed in various kinds of decaying plant and fungal material, including fruit, bark, slime fluxes, flowers and mushrooms, although a few species have switched to a parasitic or predatory lifestyle. Several *Drosophila* species, including *D. melanogaster*, *D. immigrans*, and *D. simulans*, are closely associated with humans (Parsons 1983). These and other species (*D. subobscura, Zaprionus indianus*) have been accidentally introduced around the world by human activities such as fruit transportation.

1.1.2 Laboratory use

Drosophila melanogaster is a popular experimental animal because it is easily cultured en masse out of the wild, it has a short generation time and because mutant animals are readily obtainable. In 1906 Thomas Hunt Morgan began his work on *Drosophila* and reported his first finding of a white-eyed mutant in 1910 (Ashburner 1989). He was in search of a model organism to study genetic heredity and required a species that could randomly acquire genetic mutation that would visibly manifest as morphological changes in the adult animal. His work on *Drosophila* earned him the 1933 Nobel Prize in Medicine for identifying chromosomes as the vector of inheritance for genes (Nobel.org, 2006).

Initially *Drosophila* was used in classical genetics, for example in the study of heredity, genetic linkage and genetic maps. As biological research progressed *Drosophila* has become a useful tool in molecular and developmental biology with the main focus of developmental research focussing on embryonic development, but also a great deal of interest in how various adult structures develop in the pupa, in particular the development of the eye, but also on the wings, legs and

other organs. Techniques such as in-situ hybridisation, P-element transformation and chemical mutagenesis have all advanced the use of *Drosophila* as an analytical tool (Ashburner and Bergman 2005).

Most recently research on *Drosophila* has branched out into genomics and proteomics. *Drosophila* has four pairs of chromosomes: the X/Y sex chromosomes and the autosomes 2,3, and 4 (Ashburner 1989). The fourth chromosome is much smaller and more rarely studied. The first release of the complete genome sequence was released in 2000 (Adams et al. 2000), with subsequent releases improving in quality (Ashburner and Bergman 2005). Another insect genome, the mosquito *Anopheles gambiae* (Holt, Subramanian et al. 2002) has also now been sequenced as has the recently published genome of the honey bee (*Apis mellifera*) (Consortium 2006). Other current insect genome projects include the silk moth, *Bombyx mori* (Evans and Gundersen-Rindal 2003), and a further 11 related *Drosophila* species (Flybase, 2006), This wealth of genetic information as well as the wide availability of mutants and ease of culture makes *Drosophila* an ideal model for the insects.

One genetic tool used extensively in this thesis is the UAS GAL4 expression system. This system was created to allow the targeting of gene expression in *Drosophila.* GAL4 was identified in *Sacchaomyces cerevisiae* as a gene regulator induced by galactose. The DNA binding and transcriptional activation functions of GAL4 have been shown to be separable and meticulously defined (Gill and Ptashne 1988). GAL4 is a 881 amino acid protein which binds to four 17 bp sites that define an Upstream Activating Sequences (UAS) element which is essential for the transcriptional activation of the GAL4 regulating genes. GAL4 expression was shown to stimulate transcription of a reporter gene under UAS control in *Drosophila* (Fischer, Giniger et al. 1988). The expression of GAL4 in *Drosophila* was shown to have no deleterious effects on the fly. This was followed by the development of a bipartite method for directing gene expression (Brand and Perrimon 1993). The two components of the system are the responder (the fly carrying the gene of interest controlled by UAS) and the driver (the fly expressing GAL4 in a particular pattern). The progeny resulting from crossing these two flies express the gene of interest under a transcriptional pattern controlled by the GAL4 driver. This bipartite system has the advantage of

maintaining the two parts as separate parental lines, an advantage when the responder gene is toxic, lethal or shows reduced viability. In addition the system allows the spatial and temporal expression using a vast array of GAL4 drivers strains available.

1.1.3 The role of *Drosophila* in insecticide resistance research

Drosophila melanogaster is not a pest species and is therefore not a target insect when a new insecticide is designed. New compounds are chosen for their activity against pest species such as those of the orders Lepidoptera or Coleoptera. Detailed genetic studies with pest species are often considerably more difficult than with *Drosophila* (Daly 1993). An example of this is the pest species *Helicoverpa armigera* (the Old World or African bollworm), arguably one of the most important agricultural pests with a long history of insecticide resistance (Gunning, Moores et al. 1999). It is not however an ideal insect model to study as its larvae are prone to disease even if stressed by small changes in rearing conditions; large larvae are cannibalistic and so must be reared individually, single pair crosses can have a high failure rate, and generation time is six weeks. Furthermore, genetic maps have been difficult to construct particularly because there are 31 pairs of small chromosomes (Fisk 1989).

Drosophila is a valuable tool in the study of insecticide resistance. Using *Drosophila* as a model species in the study of insecticide resistance relies on the presence of homologues of the genes identified in *Drosophila* in the pest species. One example is the gene *kdr* which has now been seen in a large range if species and orders of insects and in all cases the same small number of mutations to sodium channels have been seen (Zlotkin, 1999). It is also common in the case of metabolic resistance that a series of homologues to either one gene or a series of closely related genes will confer resistance in the same way. The Cytochrome P450, *Cyp6a1* in the housefly *Musca domestica* and its homologue *Cyp6a2* in *D. melanogaster* (Dunkov, Mocelin et al. 1997) give the same resistance profile, and even have homologous mechanisms for the over-transcription of the gene (Sabourault, Guzov et al. 2001).

1.1.4 DDT resistance in *Drosophila* as a model system

DDT resistance (*DDT-R*) in *Drosophila* is a useful model system for a number of reasons. DDT was one of the earliest and most widespread pesticides ever used. In addition, individual flies can be readily genotyped for the presence of the resistance-associated mutation using a simple Polymerase Chain Reaction (PCR) based diagnostic that exploits the insertion of the *Accord* transposable element, associated with *DDT-R*. This allows us to identify all three genotypes; *DDT-S/DDT-S* (DDT sensitive), *DDT-R/DDT-S* and *DDT-R/DDT-R* (or *SS, RS* and *RR*) in individual flies. An outstanding example of parallel evolution has been revealed as the insertion of a different transposable element in the 5' end of the *Cyp6g1 D. simulans* is also associated with insecticide resistance (Schlenke and Begun 2004). *DDT-R* is therefore a widespread, representative and current mechanism of insecticide resistance.

1.2 *CTENOCEPHALIDES FELIS*

1.2.1 Life history and ecology

The cat flea (*Ctenocephalides felis*) is a common ectoparasite of companion and wild animals. The primary host is the domestic cat, but is also the primary flea infesting dogs in most geographic areas of the world. The cat flea is capable of maintaining its life cycle on other carnivores and on the Virginia opposum. Rabbits, rodents, ruminants and man can be infested or bitten but these hosts cannot sustain a population of cat fleas. Cat flea populations are increasing and cat fleas are one of the most abundant and widespread flea species in the world (Rust, 2005).

The flea passes through four stages in its life cycle. It undergoes a complete metamorphosis at each stage (Dryden 1993). The life cycle can be completed in as little as 12 days or take as long as 174 days and is dependent upon the ambient temperature and humidity. The egg hatches between one and 10 days of being deposited on the host and falling off into the environment, depending on

the ambient temperature and humidity with ideal conditions at 70% relative humidity and 35 °C (Silverman, Rust et al. 1981). The larvae emerge from the eggs after hatching. There are three larval instars; all feed on organic debris and blood-containing faeces from adult fleas. The larvae are extremely susceptible to heat and desiccation. The mature larvae produce a sticky cocoon in which to pupate. Environmental debris may adhere to the cocoon, which helps it to go undetected and provides protection against insecticides (Dryden and Smith 1994). Pupation lasts from five to nine days. Physical pressure and changes in light, temperature, and carbon dioxide are thought to be stimuli for emergence of the adult flea. Once on the host, the flea begins feeding within seconds and becomes an obligate parasite. The flea feeds by piercing the skin of the host and inserting the tip of the labrum epipharynx to extract capillary blood. Saliva is introduced by way of the salivary pump and used as an anticoagulant (Rust and Dryden 1997). Once fleas feed and initiate reproduction they become dependent on a constant source of blood. During feeding, female fleas excrete large quantities of incompletely digested blood in long tubular coils or fine pellets. The first of multiple matings occurs on the host within eight to 24 hours. Egg production begins within 36 to 48 hours of the first blood meal, reaches maximum production between four and nine days, and may continue for more than 100 days. Egg production peaks at 40 to 50 per day and averages 27 eggs per day for the first 50 days. A single female flea may deposit over 2000 eggs during her lifetime (Dryden 1993).

1.2.2 Veterinary importance

Cat fleas are one of the most important ectoparasite of companion animals due to the transmission of other parasites and infections to dogs and cats and their owners (Rust 2005). The tapeworm *Dipylidium*, which in severe cases may cause weightloss, can be transmitted by the cat flea. A number of studies have shown that companion cats and dogs are commonly infested with *C. felis* carrying bacterial pathogens of significance to human and animal health (Shaw, Kenny et al. 2004). Most recently, a role has been described in the transmission of cat-scratch fever caused by the bacterium *Bartonella henselae* (Kelly, Rolain et al. 2005) and feline leukaemia (Vobis, D'Haese et al. 2005). In addition, flea allergic

dermatitis (FAD) can occur in 10% of infested animals and can cause severe discomfort to the animal. This in turn may lead to hair loss, dermatitis and secondary yeast and bacterial infections. Heavy flea infestations can cause anaemia due to heavy blood loss in the host animal.

1.3 INSECTICIDES

1.3.1 Classification

The classification of insecticides may use one of a number of factors. First, the stage or type of arthropod that the insecticide kills e.g. ovicides, larvicides, miticides. Second the route of entry, which includes systemics where the insecticide is ingested with food, contact poisons which enter through the cuticle, and fumigants, which enter through spiracles and tracheae. Third, the chemical nature of the compound e.g. inorganics such as arsenicals, fluorides, natural organics, oils which may be used against scale insects and aphids, botanical extracts e.g. rotenone, nicotine, pyrethrin; synthetic organics e.g. chlorinated hydrocarbons, organophosphates, carbamates, pyrethroids. Finally the mode of action of the insecticide may also be used to classify insecticides (table 1.1). Examples of the modes of action include metabolic inhibitors e.g. rotenone, neuroactive agents, which affect the transmission of nerve impulses across synapses and insect growth inhibitors e.g. juvenile hormone which inhibits moulting.

Early insecticides such as heavy metals, eg lead, mercury, arsenic and plant toxins such as nicotine have been used for many years. Chlorine based agents were developed and then with the advancement of modern chemical industry it was possible to form organochlorides. These compounds, the most famous of which was DDT were highly successful until resistance began to occur. In answer to the problem of resistance the next large class to be developed were the organophosphates, followed by the carbamates. To mimic the insecticidal activity of the natural compound pyrethrum another class of pesticides, pyrethroid pesticides, have been developed. These are non-persistent and much less

acutely toxic than organophosphates and carbamates. More recently biological insecticides have been developed, for example *Bacillus thuringiensis*, a bacterial disease of Lepidopterans and some other insects is used as a larvicide against a wide variety of caterpillars. The Bt toxin from *Bacillus thuringiensis* has been incorporated directly into plants through genetic manipulation.

Primary target site of action	Chemical subgroup or exemplifying active ingredient	Examples of Active Ingredients
Acetylcholine esterase inhibitors	Carbamates	Aldicarb
		Bendiocarb
		Carbaryl
		Carbofuran
		Methiocarb
		Methomyl
		Oxamyl
		Propoxur
		Thiodicarb
	Organophosphates	Acephate
		Azinphos-methyl
		Chlorpyrifos
		Chlorpyrifos-methyl
		Diazinon
		Dichlorvos
		Fenthion
		Isofenphos
		Malathion
		Methyl parathion
		Profenofos
		Temephos
		Terbufos
GABA-gated chloride channel antagonists	Cyclodiene organochlorines	Endosulfan
		Lindane
	Fipronil (phenylpyrazoles)	Fipronil
Sodium channel modulators	Pyrethroids	Allethrin
		d-cis-trans Allethrin
		Bifenthrin
		Cyfluthrin
		Deltamethrin
		Permethrin
		Tetramethrin
	Pyrethrins	Pyrethrins (pyrethrum)
	Methoxychlor	Methoxychlor
Nicotinic acetylcholine receptor agonists/antagonists	Neonicotinoids	Acetamiprid
		Imidacloprid
	Nicotine	Nicotine
Chloride channel activators	Avermectins, Milbemycins	Abamectin
Juvenile hormone mimics	Juvenile hormone analogues	Hydroprene
		Kinoprene
		Methoprene
	Fenoxycarb	Fenoxycarb
Compounds of unknown or non-specific mode of action	Methyl bromide	halides
	Chloropicrin	Chloropicrin
	Sulfuryl fluoride	Sulfuryl fluoride
Compounds of unknown or non-specific mode of action	Cryolite	Cryolite
Compounds of unknown or non-specific mode of action	Clofentezine	Clofentezine
	Hexythiazox	Hexythiazox
	Etoxazole	Etoxazole
Microbial disruptors of insect midgut membranes	*B.t.* var. israelensis	B.t. var. israelinsis
	B.t. var. aizawai	*B.t.* var. aizawai
	B.t. var. kurstaki	*B.t.* var. kurstaki
Inhibitors of oxidative phosphorylation, disruptors of ATP	Organotin miticides	Fentutatin oxide
	Propargite	Propargite
Inhibitors of chitin biosynthesis, type 0, Leptdopteran	Benzoylureas	Diflubenzuron
		Hexaflumuron
		Novaluron
Moulting disruptor, Dipteran	Cyromazine	Cyromazine
Ecdysone agonists/moulting disruptors	Diacylhydrazines	Halofenozide
		Methoxyfenozide
		Tebufenozide
	Azadirachtin	Azadirachtin

Table 1.1. *Insecticide Resistance Action Committee (IRAC) classification scheme for acaricides and insecticides indicating mode of action for the various groups of chemicals*

1.3.2 Mode of action

There are a wide number of modes of action by which insecticides act. A comprehensive list can be found in table 1.1. A large number of current effective insecticides target the insect's nervous system (Raymond, Berticat et al. 2001) as illustrated in figure 1.1. The major molecular modes of action of insecticides are described below. The examples discussed include acetylcholine esterase inhibitors (organophosphates and carbamates), γ-aminobutyric acid (GABA) receptors (cyclodienes and fipronil), sodium channels (pyrethroids and dichlorodiphenyl-trichloroethane (DDT)), nicotinic acetylcholine receptors (imidacloprid and spinosad), insecticides with juvenile hormone activity and midgut disruptors such as the Bt endotoxins.

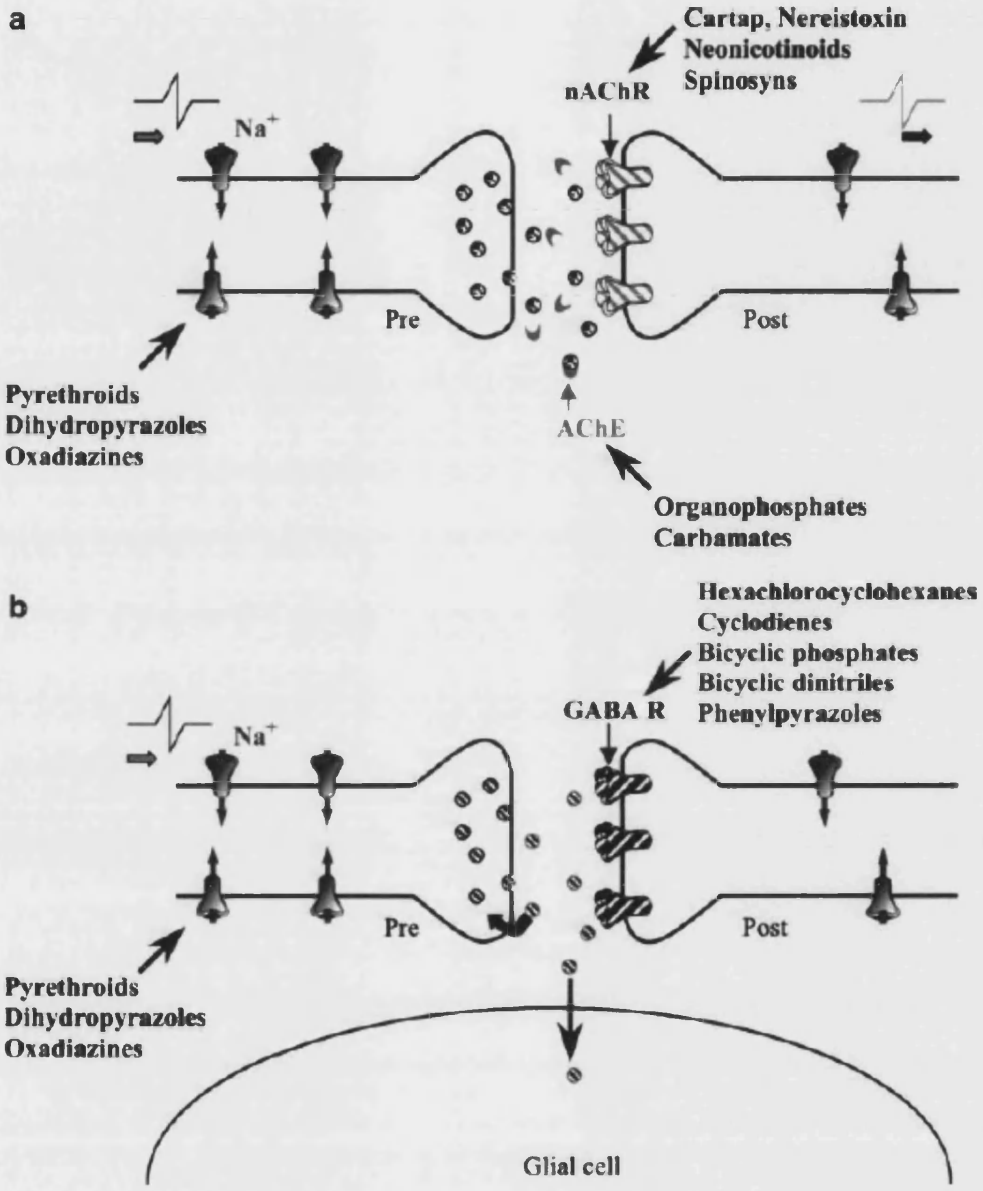


Figure 1.1 *Diagram illustrating the site of action of insecticides on the cholinergic (a) and GABAergic (b) synapses (taken from Raymond-Delpech et al 2005).*

1.3.2.1 Acetylcholine esterase inhibitors

This group includes the organophosphate (OP) and carbamate insecticides. Acetylcholinesterases (AChE) are found in the synaptic clefts of cholinergic synapses and cleave the neurotransmitter acetylcholine into its constituents, acetate and choline, limiting the size and duration of the postsynaptic potential. Insecticides that are acetylcholine esterase inhibitors prolong the length of postsynaptic potentials by allowing an accumulation of acetylcholine in the synaptic cleft. Their conformation is analogous to the natural substrate, acetylcholine, but they are partial substrates and they inactivate the enzyme by phosphorylating or carbamoylating the active-site serine (Menzozzi et al 2004). This inactivation of the enzyme blocks the degradation of acetylcholine resulting in the synaptic concentrations of acetylcholine build up and hyperexcitation of the CNS occurs. This leads to spastic muscular movements and ultimately lethal physiological disruption. In insects, the effects of OPs and carbamates are confined to the CNS, where virtually all of the cholinergic synapses are located. They often require bioactivation and must penetrate into the CNS, therefore the OPs do not have a rapid action like that of the pyrethroids. The phosphorylation of acetylcholinesterase by OPs is persistent; reactivation of the enzyme can take many hours or even days.

1.3.2.2 GABA-gated chloride channel antagonists

γ-aminobutyric acid (GABA) mediates fast chemical neurotransmission in invertebrates. Ionotropic GABA receptors (GABARs) mediate inhibitory synaptic transmission in the nervous system and at nerve-muscle junctions. GABARs belong to the cys-loop superfamily of ligand-gated ion channels. Receptors of this superfamily consist of pentamers of homologous subunits arranged around a central ion-conducting channel (Hosie, Aronstein et al. 1997). The GABARs are permeable to chloride ions and like nicotinic acetylcholine receptors (nAChRs) they possess an extracellular N-terminal region containing residues involved in ligand binding. In addition the receptors have four transmembrane domains (M1–M4) with a large intracellular loop between M3 and M4, which contains phosphorylation sites (Raymond-Delpech, Matsuda et al. 2005) (see figure 1.2).

Vertebrate GABARs fit into two categories, the $GABA_A$ receptors, which are antagonized by bicuculline and are found throughout the CNS and the bicuculline-insensitive $GABA_C$ receptors, which have a more limited distribution (Feigenspan and Bormann 1994). Both classes are blocked by the plant toxin picrotoxinin (PTX). Invertebrate GABARs also gate anion-selective channels and are antagonized by PTX, but unlike $GABA_A$ receptors, the majority of insect GABA receptors are bicuculline-insensitive, and they differ from both $GABA_A$ and $GABA_C$ receptors in their sensitivity to GABA analogues and allosteric modulators (Hosie, Aronstein et al. 1997). There are three known receptor subunit classes in *D. melanogaster*. The three known classes are encoded by three genes: *Rdl* (resistance to dieldrin); *Grd* (GABA and glycine-like receptor of *Drosophila*) and *Lcch3* (ligand-gated chloride channel homologue 3), and the subunits named accordingly, RDL, GRD and LCCH3 (ffrench-Constant and Roush 1991; Harvey, Schmitt et al. 1994).

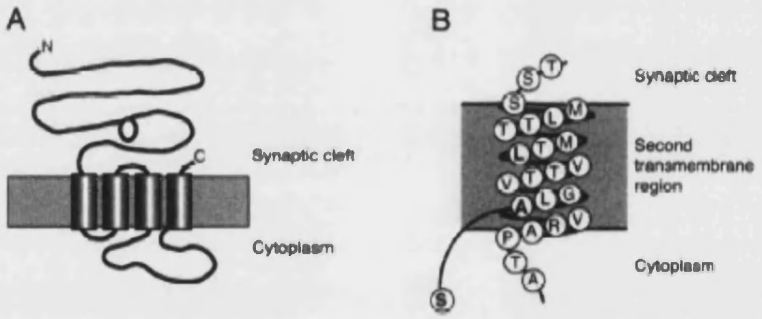


Figure 1.2 *Diagram illustrating the Drosophila Rdl-encoded GABA-receptor subunit (taken from Hosie et al.. 1997). (A) The presence a large extracellularly located N-terminal region, presumed to contain a dicysteine loop, and four transmembrane regions, is characteristic of the cys-loop family of neurotransmitter receptors. (B) The second transmembrane regions of RDL subunits are presumed to be the principal constituent of the ion-channel lining.*

The insect GABA receptor is the site of action of cyclodiene insecticides and the more recently introduced, phenylpyrazoles such as fipronil (Hosie, Aronstein et

al. 1997). These insecticides act as noncompetitive blockers of the GABA-gated chloride channel (Hainzl, Cole et al. 1998). Cloning and functional expression of RDL ionotropic GABAR of *D. melanogaster* has provided direct evidence that these GABAergic insecticides blocked responses of RDL to GABA (ffrench-Constant et al. 1993; (ffrench-Constant, Rocheleau et al. 1993; Buckingham, Hosie et al. 1994). Fipronil and PTX have different sites of action on GABARs in rat dorsal root ganglion neurones (Ikeda, Zhao et al. 2001). Although fipronil binds to the rat $GABA_A$ receptor, it is more toxic to insects than to mammals. The basis for this selective toxicity has not been resolved but actions on other ligand-gated ion channels not present in vertebrates may be a contributory factor.

It has been demonstrated that fipronil acts on insect neuronal glutamate-gated chloride (GluCl) receptors (Horoszok, Raymond et al. 2001; Raymond and Sattelle 2002). GluCls are a potential insect-specific target of fipronil because of their presence in invertebrates but not in vertebrates (Raymond and Sattelle 2002). Fipronil has been shown to block insect GABA-Cl channels. In the expressed, recombinant, wildtype, RDL_{ac} splice variant of the receptor fipronil reduces the mean time channel that the channel is open (Grolleau and Sattelle 2000). RDL receptors are also sensitive to different drugs such as picrotoxinin and BIDN (3, 3-bis-trifluromethyl-bicyclo-[2,2,1]heptane-2,2-dicarbonitrile) (Anthony, Holyoke et al. 1994; Grolleau and Sattelle 2000). Fipronil also reversibly inhibits L-glutamate-gated chloride currents recorded from the *C. elegans* GLC-3 recombinant homomeric receptor expressed in *Xenopus* oocytes (Horoszok, Raymond et al. 2001). These results indicate that GluCls and ionotropic GABARs may share some similarities in their binding sites for fipronil. Further studies on recombinant receptors and chimeras may provide further insights into the molecular basis of the fipronil's mode of action.

1.3.2.3 Sodium channel modulators

The voltage-gated sodium channels mediate the transient wave of sodium entry along the nerve axons and dendrites, carrying the action potential along the nerve. The first two sodium channel genes identified from *D. melanogaster* genomic DNA libraries were located on chromosome 2 close to the seizure (sei) locus and on chromosome X close to the paralysis (para) locus. The latter is

67% identical at the amino acid level with vertebrate neuronal sodium channel α-subunits (Raymond-Delpech, Matsuda et al. 2005). There are multiple transcript variants of para resulting from five alternative splice sites, some of which account for the high sodium channel current density observed in *D. melanogaster* embryos (Zlotkin 1999). RNA editing of *para* has been shown to add to the molecular and functional diversity of *para* gene products (Hanrahan, Palladino et al. 1999). An additional *D. melanogaster* subunit, the product of the temperature-induced paralysis (*tip-E*) locus has been found. There are at least four candidate sodium channel genes in the *Drosophila* genome (Littleton and Ganetzky 2000). Although the product of *para* alone will express to yield channel activity, a functional insect voltage-gated sodium channel has been generated by co-expression of α-like subunits PARA and TIP-E resulting in channels with increased current amplitude and rate of channel inactivation without altering the time course and voltage-dependence of the sodium current. Sodium channels on insect neurones fall into two functional categories — those that contribute to the resting membrane potential (background sodium channels) and voltage-sensitive sodium ion channels (VSSCs) involved in action potential generation (Raymond-Delpech, Matsuda et al. 2005).

Several natural and synthetic toxins act at distinct sites on sodium channels and modify their function. These include the pyrethrins and the synthetic pyrethroids as well as DDT. Pyrethrins are derived from the plant extract pyrethrum isolated from chrysanthemum flowers (Casida, Gammon et al. 1983). Their stability in natural light is poor and so the structure was modified to produce more stable compounds that could be used as insecticides (Shafer, Meyer et al. 2005). Pyrethroids are synthetic pyrethrins categorised into two types based on the toxic effects on rats (Bloomquist 1993). Both type I and type II inhibit the VSSCs. Type II have also been shown to affect the neurotransmitter GABA (Narahashi, Frey et al. 1992). Pyrethroids slow the activation, or opening, of VSSCs. In addition, they slow the rate of VSSC inactivation (or closing) and shift the membrane potential at which VSSCs activate (or open) (Narahashi, Frey et al. 1992). This results in the sodium channels opening at more hyperpolarized potentials and prolongs the opening of the channel, allowing more sodium ions to cross and depolarize the neuronal membrane. In general, type II compounds delay the inactivation of VSSCs substantially longer compared with type I

compounds (Shafer, Meyer et al. 2005). Type I compounds prolong channel opening long enough to cause repetitive firing of action potentials (repetitive discharge), whereas type II compounds hold open the channels for such long periods of time that the membrane potential ultimately becomes depolarized to the point at which generation of action potentials is not possible. This is known as depolarization-dependent block. (Shafer, Meyer et al. 2005).

DDT is a highly hydrophobic solid widely used in the 1940s and 1950s, in particular to control the vectors of typhus and malaria. Use of DDT declined as resistance occurred and in the 1970s and 1980s use of DDT was banned due to concerns about toxicity to bird populations and the persistence of the compound in the environment (WHO, 2005). This is largely a consequence of its extreme level of insolubility (1-2 ppb) in water when compared to lipid solubility. This favours the storage and accumulation of the compound in animal membranes (Zlotkin, 1999). DDT use is still advocated for malaria vector control due to the high levels of mortality caused by malaria, however use is strictly governed by the protocols of the Stockholm Convention on Persistent Organic Pollutants (POPs) and use for disease vector control is negligible (UNEP, 2001). The mode of action of DDT is similar to that of pyrethroids (Vijverberg, van der Zalm et al. 1982). The compound induces intense repetitive activity in sense organs and in myelinated nerve fibres resulting in the death of the insect.

1.3.2.4 Nicotinic acetylcholine receptor agonists

The nicotinic acetylcholine receptors (nAChRs), like the GABARs, are members of the cys-loop superfamily of ionotropic neurotransmitter receptors. In the case of nAChRs, the integral ion channel becomes transiently permeable to cations following the binding of Acetylcholine (Ach) (Unwin 1993). The nAChRs are made up of five homologous polypeptide subunits. These polypeptide subunits share a common organization, a long extracellular N-terminal domain containing residues that contribute to the ACh binding site and four membrane spanning regions (M1-4) (Karlin 2002). There are two classes of subunit, the α subunits, which are defined by the presence of two adjacent cysteine residues in loop C, one of the several extracellular regions (loops A-F) that contribute to the ACh

binding site. The non-α subunits do not possess this Y·CC motif (Karlin 2002). The *D. melanogaster* nAChR gene family is the smallest to date, with only 10 members (Sattelle, Jones et al. 2005). There is still a wide diversity within this family however due to alternative splicing of four of these 10 subunits and RNA editing which alters key amino acids in the receptor (Sattelle, Jones et al. 2005).

Two classes of closely related compounds act on the nicotinic acetylcholine receptor (nAChR). These classes are the neonicotinoids, including imidacloprid and the nicotinoids such as epibatidine, nicotine and desnitro-imidacloprid. The two classes are differentiated by their structure, their mode of action as agonists of the nAChRs of invertebrates and vertebrates and their ionisation state under physiological pH (Tomizawa, Lee et al. 2000; Kagabu 2003). The neonicotinoids are not ionised and are selective for insect nAChRs where as nicotinoids are ionised and are more active against vertebrate nAChRs (Tomizawa and Casida 2003). Imidacloprid, a neonicotinoid insecticide, mimics the action of the neurotransmitter, acetylcholine (ACh). The neuron is continually stimulated by imidacloprid, the end result is similar to that caused by cholinesterase inhibitors. The over stimulation of the nervous system leads to poisoning and death. The selectivity of imidacloprid for insect nAChRs makes it an ideal candidate for an insecticide and consequently imidacloprid is one of the worlds biggest selling insecticides with sales of $455 US million in 1999 (Maienfisch, Angst et al. 2001).

1.3.2.5 Juvenile hormone mimics

Growth and development in insects are regulated by ecdysteroids and the sesquiterpenoid juvenile hormones (JHs) (Wigglesworth 1964; Nijhout 1981). JH is synthesized and secreted by the corpus allatum and appears to be unique to insects (Wilson 2004). Juvenile hormone and the steroid 20-hydroxyecdysone (20E) are involved in the regulation of reproductive maturation in the adult insect. The moulting process is initiated by an increase in 20E and is completed following the decline of 20E and the release of eclosion hormone (Nijhout 1981). The larval-pupal moult is induced with moulting levels of ecdysteroids in the presence of JH. Juvenile hormone is required to prevent premature development of the imaginal disks in holometabolous insects. Finally, for the pupal-adult

transformation, a steady increase of ecdysteroid titers at low levels commits the tissues to adult differentiation, and at higher levels, it elicits that differentiation. Growth and development in insects are very well orchestrated by 20E, JH, eclosion hormone, and other neurohormones. Any interference in the homeostasis of one or more of these hormones with exogenous sources of the hormones or with synthetic analogs (agonists or antagonists) results in the disruption or abnormal growth and development of the target insect (Dhadialla, Carlson et al. 1998).

Synthetic analogues of the juvenile hormone (JHAs) are used as an insecticide, preventing the larvae from developing into adult insects (Wilson 2004). At high levels of JH, larva can still moult, but the result will only be a bigger larva, not an adult, thus breaking the reproductive cycle. JHAs are very insect specific with a limited predictability of the efficacy against various targeted species. The JHA methoprene is very effective against dipteran insects but less so against lepidopteran insects (Henrick, Willy et al. 1975; Amos, Williams et al. 1978). Studies on *Drosophila* have shown that methoprene mimics the action of JH III acting as a JH agonist (Wilson 2004). Methoprene, is approved by WHO for use in drinking water cisterns to control mosquito larvae and has been widely used in mosquito control, as well as in the control of dipteran pests of livestock (Glare and O'Callaghan 1999). The agricultural use of early juvenile hormone analogues was initially limited by a lack of outdoor stability, limited insect control spectrum, and their slow toxic action (Dhadialla, Carlson et al. 1998). Development of insecticides with an insect moulting hormone mode of action has also been pursued, although currently without much success, largely due to their structural complexity and the relative instability of the steroid nucleus (Dhadialla, Carlson et al. 1998).

1.3.2.6 *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) has been widely used in pest control for decades due to high target specificity and a low persistence in the environment, Bt has been used in particular to control lepidopterous larvae and was supplemented recently by strains selected for potency on various target pests e.g. *Bacillus thuringiensis*

subsp. aizawai and *B. thuringiensis* subsp. *kurstaki* for Lepidoptera (Wang, Zhang et al. 2006) , *B. thuringiensis* subsp. *israeliensis* for mosquito control (Federici, Park et al. 2003), and *B. thuringiensis* subsp. *tenebrionis* for Coleoptera (Hussein, Habustova et al. 2005). There have been over 150 insecticidal crystal (Cry) proteins discovered in *Bacillus thuringiensis* and *Bacillus cereus* (Schnepf, Crickmore et al. 1998). *B. thuringiensis* synthesizes the insecticidal crystalline inclusions containing δ-endotoxins during sporulation (Liang, Patel et al. 1995). Once solubilised, the δ-endotoxin are thought to form pores in the epithelium plasma membrane of the insect larvae midgut (Liang, Patel et al. 1995). The first two steps in the channel formation process for an activated toxin are binding to the membrane and insertion into the membrane. The toxin binds to specific receptors on the apical brush border of the midgut microvillae of susceptible insects (Schnepf, Crickmore et al. 1998). Experiments on tissue culture cells have shown that under certain conditions, including high-toxin concentrations, long incubation times, and relatively low pHs, Cry toxins insert into membranes and form pores. These pores are either considered to be a large lytic pore that is not specific for particular ions or an ion-specific channel that disrupts the membrane potential but does not necessarily lyse midgut epithelial cells (Schnepf, Crickmore et al. 1998). This unique mode of action of the Cry proteins results in the high selectivity for the target pest (Whalon and Wingerd 2003). A more recent development is the development of transgenic crops expressing the Cry toxins. Currently crops expressing Cry proteins for the control of pests include tobacco, cotton, potatoes, and corn (Whalon and Wingerd 2003). They have already been used on several million acres and greatly reduced the amounts of certain synthetic chemical insecticides required for effective pest control (Betz, Hammond et al. 2000).

1.4 INSECTICIDE RESISTANCE

1.4.1 Definition

The World Health Organisation define resistance as: "Development of an ability in a strain of an organism to tolerate doses of a toxicant that would prove lethal to the majority of individuals in a normal (susceptible) population of the species"

(Zlotkin, 1999). Insecticide resistance occurred long before the use of insecticides by man. Some plants have evolved to produce a range of toxic compounds (e.g. nicotine) driving the evolution of new forms of resistance to these compounds (Feyereisen, 1999) in the target insect. There are a number of examples of the evolution of insecticidal compounds in a plant followed by the ability of insects to survive exposure to this compound allowing them to feed on the plant and so out-compete competitors by being able to survive in a niche environment. One example of this is the over-expression of the cytochrome P450 CYP4M3 in *Manduca sexta* (Snyder et al., 1995). This enables the insect to survive in the presence of nicotine and therefore is possibly, why this particular Lepidoptera is capable of living on plants that produce such a potent natural pesticide. Resistance to insecticides was first documented in 1914 by A.L. Melander in the Journal of Economic Entomology. He described scale insects, still alive, under a "crust of dried spray" of an inorganic insecticide. It has been estimated that insecticide resistance in the United States adds $40 million to the total insecticide bill in additional treatment costs or alternative controls (IRAC 2006). Better management of pesticides by farmers and the crop experts assisting them, could reduce this bill and lead to more effective, more efficient use of products (IRAC 2006).

1.4.2 Mechanisms

There are four general mechanisms of insecticide resistance (Soderlund and Bloomquist, 1990). The two major mechanisms that will be described in detail here are firstly target site resistance where changes to the target site of the insecticide, such as the receptor the insecticide is unable to bind to its target and so is unable to have an effect. A second mechanism is metabolic resistance, where an enzyme that is capable of rendering a compound harmless to the insect is up-regulated and so the insecticide is unable to reach the target site (Taylor and Feyereisen, 1996). Target site mutations are generally associated with extremely high levels of resistance compared with metabolic resistance, where the rate of detoxification is a limiting factor. In addition to these mechanisms there can be a physiological change in the insect, such as a change in the midgut wall or cuticle that makes it more difficult for the insecticide to enter the insect

(Taylor and Feyereisen, 1996). Finally a minor form of resistance is named behavioural avoidance. In this form of resistance, an insect will not feed on areas sprayed with insecticide. This phenomenon has been demonstrated with malathion in *D. melanogaster* but it seems to be a minor form of resistance (Pluthero and Threlkeld, 1984).

1.4.2.1 Target site resistance

1.4.2.1.1 GABA receptor

The GABA-receptor-subunit *Resistance to dieldrin (Rdl)* gene was cloned from a field-isolated *D. melanogaster* mutant that was resistant to the cyclodiene insecticide dieldrin (Zhang, ffrench-Constant et al. 1994). Mapping of dieldrin resistance in *D. melanogaster* showed that resistance was conferred by a single gene on chromosome III at map position 66F (ffrench-Constant and Roush 1991). The generation and analysis of chromosomal re-arrangements, e.g. deletions and inversions, pinpointed the open reading frame responsible, *Rdl* (ffrench-Constant and Roush 1991). The gene encodes a subunit of the insect GABA receptor (ffrench-Constant, Rocheleau et al. 1993). These subunits show 30-38% homology to vertebrate GABA receptors (Hosie, Aronstein et al. 1997). The encoded RDL subunit assembles with other GABA receptor subunits to form the target site of the cyclodiene and phenylpyrazole insecticides (ffrench-Constant, Anthony et al. 2000). Target site resistance is associated with point mutations in *Rdl* that predict replacement of alanine302 with either a serine or a glycine as seen in figure 1.3 (ffrench-Constant, Anthony et al. 2000). The replacement is thought to be located in M2, the putative channel-lining domain of RDL subunits. M2 is encoded by exon 7, which is not alternately spliced (Hosie, Aronstein et al. 1997). Biophysical analyses have proposed the model that alanine302 is unique in that it both occupies the drug-binding site directly and also allosterically destabilizes the drug-preferred desensitized state of the receptor (Zhang, ffrench-Constant et al. 1994).

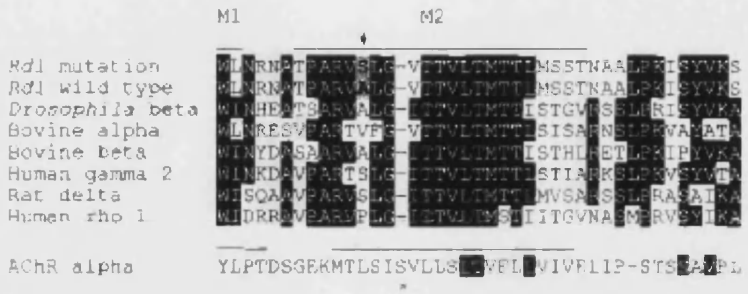


Figure 1.3. *The alignment of a number of GABA receptor amino-acid sequences. Amino acids found in the majority of the nine sequences in black. The high level of homology between receptors is clear (taken from ffrench-Constant et al 1993).*

Mutations replacing alanine302 are highly conserved throughout a range of different insect orders (ffrench-Constant, Anthony et al. 2000) indicating parallel evolution of the resistance mutation. This includes a number of pest species such as the German cockroach, *Blattella germanica* in which a substitution from alanine to serine has been shown to correlate with 1,270- and 2,030-fold increase in resistance to dieldrin (Hansen, Kristensen et al. 2005). In the mosquito *Anopheles gambiae* a subsitution at position 302 from alanine to glycine has been is genetically linked to resistance to dieldrin (Du, Awolola et al. 2005). In addition the susbstitution has been identified in *Drosophila simulans* (Sturtevant), the housefly, *Musca domestica*, the American cockroach, *Periplaneta americana* (L), the red flour beetle, *Tribolium castaneum* (Hbst), the mosquitoes *Aedes aegypti* and *Anopheles stephensi*, the coffee berry borer, *Hypothenemus hampei* Ferrari, the whitefly, *Bemisia tabaci* Gennadius and the peach potato aphid, *Myzus persicae* (ffrench-Constant, Anthony et al. 2000). In contrast with other insects, *M. persicae* clones have been shown to possess multiple *Rdl* alleles (A, G, and two separate serine alleles S and S'), which are found in separate loci (Anthony, Unruh et al. 1998). In the first locus both the alanine302 to serine and alanine302 to glycine replacements present at the first Rdl locus. At the second Rdl-like locus it appears that there is always a serine in position 302. Resistance to the compound endosulfan is correlated with variation at the first locus only. This second locus is either thought to represent the result

of a gene duplication event unique to aphids, or that it may correspond to a putative subunit presumed to coassemble with RDL (Anthony, Unruh et al. 1998). The number of independent origins of *Rdl* resistant alleles in a species is thought to depend on the life history and migration of the species. *Drosophila* shows evidence of a single mutational event which has spread globally, consistent with the highly levels of migration in these species. The red flour beetle *Tribolium castaneum*, with its low levels of dispersion shows evidence of multiple origins of Rdl resistance (Hosie, Aronstein et al. 1997; Andreev, Kreitman et al. 1999).

Rdl resistant insects also show temperature sensitive paralysis (ffrench-Constant, Steichen et al. 1993). After being placed at 38 °C, flies are unable to take off from the substrate when returned to room temperature, whereas wildtype flies fly away immediately. This phenotype has been observed in other nervous system mutants such as those of the voltage-gated sodium channel gene, $para^{ts}$ (temperature sensitive paralysis), the site of action of DDT and pyrethroids (Wu and Ganetzky 1980). The temperature sensitive phenotype of *Rdl* is semidominant, as is the case for the insecticide resistance phenotype. Genetic deletion of the *Rdl* locus was shown to be lethal in *Drosophila.* Flies that are homozygous for a deletion removing *Rdl* die as late embryos and do not appear to be able to hatch from the egg (Stilwell, Rocheleau et al. 1995).
This mutation was originally associated with cyclodiene resistance and although these compounds are used less frequently now, selection for the mutation continues due to the conferred cross-resistance to other compounds including fipronil (Cole, Nicholson et al. 1993; Hosie, Baylis et al. 1995; Hansen, Kristensen et al. 2005; Kristensen, Hansen et al. 2005). Electrophysiological studies on RDL receptors expressed in *Xenopus* oocytes, (Hosie, Baylis et al. 1995) have shown that the A301S mutation, strongly reduced the potency of fipronil as well as BIDN and picrotoxin (PTX) (ffrench-Constant, Rocheleau et al. 1993; Grolleau and Sattelle 2000). RDL wild-type receptors and mutated RDL(A302S) receptors in *Heliothis virescens* showed a similar sensitivity to fipronil (Wolff and Wingate 1998). These contradictory results could be explained by differences in experimental conditions however fipronil is also though to act on the L-glutamate-gated chloride channels, which may help to explain this unexpected result.

1.4.2.1.2 Sodium channel

Target-site resistance to pyrethroids was first characterized as knockdown resistance (*kdr*) in house flies (Busvine 1951). *Kdr* confers resistance to all known pyrethroids and DDT (Soderlund and Knipple 2003). A second resistance trait was identified in the housefly and shows greater resistance than *kdr* to DDT and some pyrethroids (Soderlund and Knipple 2003). Cloning of the major subunit of the channel relied on the identification of a gene from a temperature sensitive paralytic ($para^{ts}$) Drosophila mutant (Loughney, Kreber et al. 1989). Genetic studies were performed that showed linkage between the *kdr* phenotype and the para homologue in the house fly (Williamson, Denholm et al. 1993; Knipple, Doyle et al. 1994). Comparison of sequences from wildtype, *kdr* and super *kdr* strains identified a mutation of leucine to phenylalanine at position 1014 (L1014F) in *kdr* and *super kdr* flies and an additional methionine to threonine at residue 918 (M918T) in *super kdr* strains (Ingles, Adams et al. 1996; Miyazaki, Ohyama et al. 1996; Williamson, Martinez-Torres et al. 1996). When house fly sodium channels with these resistance mutations in are expressed in *Xenopus* oocytes, a reduced sensitivity to the pyrethroids cismethrin and cypermethrin is observed (Smith, Ingles et al. 1998; Lee, Smith et al. 1999). Both mutations are thought to block binding of pyrethroids to the voltage-gated sodium channel pore (Williamson, Martinez-Torres et al. 1996). Similar to the *Rdl* situation, subsequent analysis of a wide range of pest insects has also revealed equivalent mutations at similar positions in the channel including *A. gambiae, Plutella xylostella, M. persicae and C. pipiens* (Soderlund and Knipple 2003). The diversity of mutations capable of conferring knockdown resistance results in the necessity for careful design of molecular diagnostic assays.

1.4.2.1.3 Acetylcholinesterase

The first case of AChE with reduced sensitivity to pesticides was reported by Smissaert (Smissaert 1964). AChE modifications have been described since then in a number of species including *D. melanogaster* (Mutero, Pralavorio et al. 1994), *C. pipiens* (Weill, Lutfalla et al. 2003) and *M. domestica* (Walsh, Dolden et al. 2001). Quantitative and qualitative changes of acetylcholinesterase can affect

the sensitivity of insects to acetylcholine. The amount of acetylcholinesterase in the central nervous system is important in *D. melanogaster.* Flies that overexpress the enzyme are more resistant than wild-type flies that express low levels of acetylcholinesterase. The overproduction of acetylcholinesterase outside the central nervous system also protects against organophosphate poisoning. Flies producing a soluble acetylcholinesterase, secreted in the haemolymph, are more resistant to organophosphates. Resistance can also result from a qualitative modification of acetylcholinesterase. A number of mutations have been identified in resistant *Drosophila* strains (Menozzi, Shi et al. 2004). Each of these mutations gives a different pattern of resistance and different combinations of these mutations can lead to highly resistant enzymes (Fournier, Berrada et al. 1996). The publication of the *A. gambiae* genome and work with *C. pipens* and *A. aegyptii* led to the discovery of a second AChE in insects, Ace-1 (Weill, Lutfalla et al. 2003 Weill, Fort et al. 2002). This gene is present in a range of insects although appears to have been lost from some diptera relatively recently. This loss appears to have occurred after the split between the mosquito and fly (Weill, Fort et al. 2002). The exact cause of resistance in strains with two copies of AChE is unknown as yet. The discovery of a second *Ace* gene highlights the problem of using only one insect species as a model for all other insects.

1.4.2.2 Metabolism

1.4.2.2.1 Cytochrome P450 enzymes

Cytochrome P450 monooxygeases are found within a large number of organisms from bacteria to mammals (Berge, Feyereisen et al. 1998). The function of these enzymes in insects is wide-ranging and includes the biosynthesis and degradation of ecdysteroids and juvenile hormones, important in insect growth, development and reproduction (Feyereisen 1999). In addition P450s metabolise pheromones and in herbivorous insects they detoxify toxic chemicals produced by host plants (Danielson, MacIntyre et al. 1997). Finally, P450 enzymes metabolise insecticides, either for bioactivation or detoxification. The substrate

specificity of P450 enzymes can vary with some enzymes displaying a very broad range of substrates (Feyereisen 1999).

P450s are so called because of their absorbance peak at 450nm in the optical spectrum of the carbon monoxide bound reduced form of the enzyme (Hodgson and Tate 1976). The nomenclature introduced originally by Nebert et al. (1987) proposes that all members of a family share >40% identity at the amino acid sequence level, and members of a subfamily share >55% identity (Nebert, Adesnik et al. 1987). P450 enzymes bind molecular oxygen and receive an electron from nicotinamide-adenine dinucleotide phosphate (NADPH). An oxygen atom is bound to the substrate and a molecule of water formed (Berge, Feyereisen et al. 1998). This can be represented as follows:

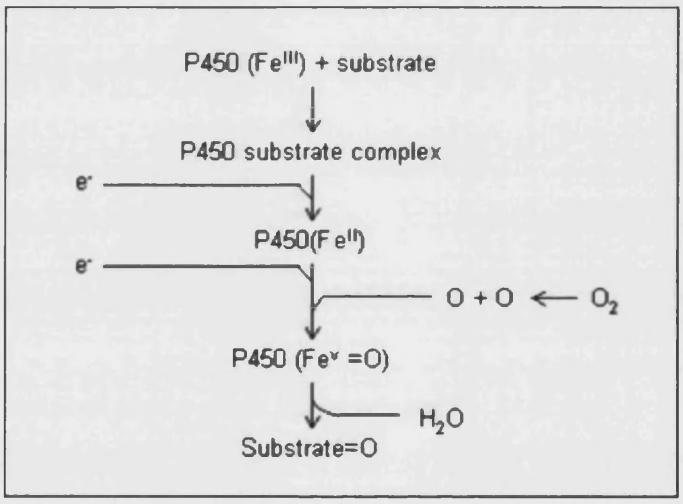


Figure 1.4 *A simplified diagram indicating the reaction catalysed by P450s.*

P450s are believed to have arisen from a precursor similar to CYP51, a lanosterol 14 a-demethylase, a P450 found in all clades. It has been proposed that P450s arose before the diversification of prokaryotes and eukaryotes (Feyereisen 1999). Virtually all P450 families fit the proposed evolutionary tree, with a few cases of parallel inheritance. Simple unicellular organisms have only a small number of identified cytochrome P450s (Nelson 1999). In more complex multicellular organisms, larger numbers of P450s have evolved as more complex

biochemical interactions between a wider range of molecules have evolved. Bacterial P450s are soluble whereas eukaryotic P450s are membrane-bound enzymes residing either in the endoplasmic reticulum or the inner mitochondrial membrane (Tijet, Helvig et al. 2001). Clusters of related P450 genes are evidence of gene duplication events in the evolution of insect genomes. It is unclear whether the duplication of P450s drove the evolution of complex organisms or whether they are by-products of evolution that facilitated more complex biochemical pathways (Nelson 1999). Some species have a very large numbers of Cytochrome P450 genes. The genome of *Arabidosis thaliana* contains over 350 predicted P450s genes. A comparison with the rice genome sample sequence suggests that this number would appear to be about the standard for both monocotyledon and dicotyledon plants (Nelson 1999). It is thought that plants have a high number of P450s due to a higher biochemical complexity when compared with animals. A large range of toxins may deter animals from feeding on the plant, a necessity due to the lack of movement by plants (Zangerl 2003).

Enhanced P450 activity has been well established as a mechanism for insecticide resistance towards most classes of insecticide. As a consequence it is an important mechanism for study (Berge, Feyereisen et al. 1998). P450 based resistance can be characterised by the loss of resistance by treatment with P450 inhibitors such as piperonyl butoxide or by comparing the NADPH dependant metabolism of the insecticide in resistant and susceptible insects (Berge, Feyereisen et al. 1998). A number of Cytochrome P450 monoxygenases have been implicated in insecticide resistance. The P450 *Cyp6a1* gene in the house fly *Musca domestica,* is constitutively overexpressed in a number of insecticide-resistant strains; (Feyereisen, Koener et al. 1989; Carino, Koener et al. 1994). CYP6A1 is overproduced in the Rutgers strain *M. domestica* (Sabourault, Guzov et al. 2001). CYP6A1 has been shown to efficiently metabolise diazinon when purified and reconstituted. The elevated CYP6A1 mRNA levels are not the result of gene amplification (Cohen, Koener et al. 1994) and so are likely to be due to increased transcription. Constitutive overexpression of CYP6A1, located on chromosome 5, requires the presence of at least one copy of chromosome 2 from Rutgers strain, implying the presence of a trans-regulator (Cohen, Koener et al. 1994). Increased *Cyp6d1* has been

associated with pyrethroid resistance in the resistant housefly strain LPR, suggesting that over-expression of this P450 is important in resistance (Tomita, Liu et al. 1995; Tomita and Scott 1995; Liu and Scott 1998).

The genome of *D. melanogaster* contains 80 known functional P450 enzymes and 7 apparent pseudogenes. Humans, mice and *C elegans* all have around 50-90 P450s (Nelson 1999). In *D. melanogaster* mutations in *Cyp6a2* have been shown to be involved in DDT resistance with one mutation affecting the metabolism of DDT when expressed in-vitro (Maitra, Price et al. 2002; Amichot, Tares et al. 2004). In addition to *Cyp6a2*, several other Cyp6 genes have been implicated in DDT resistance in *D. melanogaster* (Maitra, Dombrowski et al. 1996). A cluster of Cyp6 genes including *Cyp6a8* and *Cyp6a9* were identified in this cluster. *Cyp6a8* is more transcriptionally active in a DDT resistant strain, 91-R compared with the DDT susceptible strain 91-C and this is thought to be due to a mutation in a repressor gene in the resistant strain (Maitra, Price et al. 2002). *Cyp12a4* expression in *D. melanogaster* midgut and Malpighian tubules has been shown to confer lufenuron resistance, but not result in cross resistance to other insecticide classes (Bogwitz, Chung et al. 2005).

Cyp6g1 in *D. melanogaster* is associated with resistance to DDT, the use of which is now much reduced (Roberts, Laughlin et al. 1997), and cross-resistance to a range of other insecticides (Daborn, Boundy et al. 2001; Daborn, Yen et al. 2002; LeGoff, Boundy et al. 2003). *DDT-R* has been shown to be associated with the overtranscription of the P450 gene *Cyp6g1* (Daborn, Boundy et al. 2001). This is described in more detail in chapter 2. When a field derived resistant strain WIS1, only over-expressing *Cyp6g1* is subjected to a number of different selection regimes in the laboratory, several other P450s are up-regulated (Le Goff, Boundy et al. 2003). With intense laboratory selection of WIS1 with DDT, a second P450 gene *Cyp12d1* was up-regulated. When both of these genes were excluded by genetic recombination, further selection led to the up-regulation of a third P450 gene, *Cyp6a8*. This demonstrates that selection in the laboratory will select for other P450 alleles and further indicates that many different P450s have the capability to confer DDT resistance.

1.4.2.2.2 Esterases

Esterase based resistance either involves the rapid sequestering of the insecticide and slow metabolism (commonly over a broad range of insecticides) or narrow spectrum resistance of insecticides all containing a ester bond (Hemingway 2000). Esterases involved in sequestering are commonly found at increased levels in resistant insects due to gene amplification (Vaughan, Rodriguez et al. 1995). Gene amplification is well documented in *M. persicae, C. quinquefasciatus, C. pipiens* (Mouches, Pasteur et al. 1986; Field and Devonshire 1998) among others. Esterases which produce resistance through increased metabolism are commonly found to have point mutations which increase the structural specificity of the insecticide. In aphids there are two esterases commonly amplified in resistant insects named E4 and FE4. Both occur in susceptible insects but are amplified in resistant insects resulting in increased esterase production in the resistant insects. Amplified levels of E4 are found in linkage disequilibrium with a *kdr*-type pyrethroid resistance mechanism, possibly reflecting selection favouring multiple resistance mechanisms, chromosomal linkage or possibly due to the parthenogenic reproduction of aphids (Devonshire, Field et al. 1998). The E4 esterase occurs at a single chromosomal location but the FE4 genes have multiple sites of insertion. Amplification of genes which can cause resistance has also been seen in the mosquito *C. pipens* where the amplification of a B1 esterase is responsible for organophosphate resistance (Mouches, Magnin et al. 1987; Mouches, Pauplin et al. 1990). In *Culex* at least eight esterase containing amplicons have been identified. In *L. cuprina* the E3 malathion carboxyesterase has a point mutation in the gene LcαE7 (McKenzie, Parker et al. 1992). This confers >130-fold resistance to malathion in the resistant strain Woodside 5.2, compared to the susceptible strain, LS2 (Campbell, Yen et al. 1998).

1.4.2.2.3 Glutathione-S-Transferases

Glutathione-S-Transferases (GSTs) are capable of conjugating reduced glutathione (N-(N-L-gamma-glutamyl-L-cysteinyl)glycine or GSH) to an insecticide or its primary toxic metabolic products (Hemingway 2000). The GST

mechanism can work in a linkage disequilibrium with other resistance mechanisms such as esterase based resistance mechanisms. An example of this is in *Anopheles subpictus.* The majority of cases of GST based resistance have been identified in organophosphate resistant houseflies (Clark, Shamaan et al. 1987; Syvanen, Zhou et al. 1994). There is also evidence that GSTs are involved in the resistance to pyrethroids in the planthopper *N. lugens* (Hemingway 2000). A small number of GST are able to dehydrochlorinate insecticides such as DDT, acting as a cofactor in the reaction, rather than the more common role of a conjugate (Clark and Sharmaan 1984). This is thought to be the most common mechanism for DDT resistance in mosquitoes (Hemingway 2000). The upregulation of GSTs in resistant *A. aegypti* are due to an uncharacterised trans acting regulator.

1.4.3 Managing resistance

The ultimate strategy to avoid insecticide resistance is to prevent it occurring in the first place. How quickly resistance develops depends on several factors, including how quickly the insects reproduce, the migration and host range of the pest, the crop protection products persistence and specificity, and the rate, timing and number of applications made. Resistance increases fastest in situations such as greenhouses, where insects or mites reproduce quickly, there is little or no immigration of susceptible individuals, and the grower may spray frequently (IRAC, 2006). The key to managing resistance is to reduce selection pressure. High pesticide doses rapidly select for resistance. The Insecticide Resistance Action Committee (IRAC) recommends a number of resistance management guidelines to keep pesticides for crop pests and vectors working effectively and keep costs down (IRAC, 2006). The number of treatments and the concentration of treatments may be varied in practices considered to be good resistant management (McKenzie 1996). In theory, using low frequency doses of insecticide to increase survivorship of susceptibles can reduce selection for resistance.

Methods for preventing resistance include minimizing insecticide use and avoiding persistent applications. Ideally, an effective insecticide should be applied at a concentration high enough to kill all individuals in a population, but

disappear quickly from the environment, so that the insecticide residues do not persist at a concentration that will kill only susceptible individuals. In addition it is advised that mixing insecticides is to be avoided. A mixture of two insecticides may provide better short-term control than either insecticide used alone, but there is a danger in the long-term use of insecticide mixtures. If individuals exist with resistance mechanisms to both chemicals, then continued use of the mix will select for these multiply resistant pests. A further strategy is to use long-term rotations of chemicals with differing modes of action. Each effective insecticide is used for at least one generation of the pest before rotating to a different insecticide. If two insecticides are used within the same pest generation, the selection effect will be the same as using a mix. By using pesticides with non-specific modes of action, such as insecticidal soaps and horticultural oils it is therefore unlikely that resistance will occur to both of these due to more broad modes of action. In addition the parallel use of biological control with the chemical control methods can increase mortality without selecting for resistance, and may conserve the effectiveness of insecticides. Effective natural enemies include predators, parasitoids, and/or insect pathogens.

The problem with chemical mixing and rotation strategies is that they assume a reduction in the frequency of the resistant genotype in the absence of the insecticide. This assumes a cost to resistance. There is evidence to suggest that there is not necessarily a cost to resistance. *Rdl* frequencies in *Drosophila* populations were recorded at 1%-10% in the apparent absence of cyclodiene selection in an orchard in New York (Aronstein, Ode et al. 1994). The prevalence of the *DDT-R* associated *Accord* element in non-African populations is estimated to be 85-100%. DDT use was widely withdrawn in the 1980s. The persistence of the *Accord* element indicates a lack of any fitness cost.

1.5 Aims of thesis

1. To study the spatial pattern of expression of *Cyp6g1* in resistant and susceptible flies

2. To investigate the effect of the knock down of *Cyp6g1* expression on DDT resistance and metabolism

3. To measure the costs of resistance in a DDT resistant strain

4. To investigate the effect of *Cyp6g1* over expression on fecundity

5. To study *Cyp6g1* expression in sperm

6. To develop and apply a diagnostic test for *Rdl* in *C. felis*

CHAPTER 2

The role of *Cyp6g1* in DDT resistance

2.1 INTRODUCTION

2.1.1 DDT resistance and *Cyp6g1*

The extensive literature on *Drosophila* cytochrome P450 genes suggests that a large number of different P450s are involved in insecticide resistance (ffrench-Constant, Daborn et al. 2004). The study of DDT resistance in *Drosophila* has indicated that one Cytochrome P450, *Cyp6g1* is an important metabolic enzyme in insecticide resistance (Daborn, Boundy et al. 2001). DDT resistance in *Drosophila* was mapped by Ogita to the to right arm of chromosome 2 of *Drosophila*, in a single dominant locus at 65 cM (Ogita 1961). In addition Ogita showed the DDT resistant strains were also negatively cross resistant to phenylthiourea (Ogita 1960). This is assumed to be due to the metabolism of phenylthiourea to the toxic compound, phenylurea by the products of the resistance alleles. The position 65 on chromosome 2 was subsequently found to be associated with increased P450 activity in the DDT resistant strain Hikone-R (Hallstrom and Blanck 1985). The region contains a cluster of P450 genes, comprising *Cyp6g1, Cyp6g2* and *Cyp6t3* (Daborn, Boundy et al. 2001) and of these *Cyp6g1* is overexpressed in a large number of field strains (Daborn, Yen et al. 2002). Microarray analysis of mRNA expression in two DDT resistant strains, Hikone-R and WC2, compared with two DDT susceptible strains, Canton-S and Oregon-RC, showed *Cyp6g1* to be over-transcribed in both resistant strains compared to the susceptible strains.

Some researchers dispute the involvement of just this one Cytochrome P450 in DDT resistance (Festucci-Buselli, Carvalho-Dias et al. 2005; Pedra, Festucci-Buselli et al. 2005). One study of the relationship between mRNA expression and the LC50 of resistant and susceptible strains showed no direct correlation between *Cyp6g1* mRNA/protein expression levels and the LC50s of the small number of strains tested (Festucci-Buselli, Carvalho-Dias et al. 2005). The highest levels of *Cyp6g1* expression were found in Hikone-R, but only intermediate DDT resistance levels. The strain 91-R showed the highest resistance to DDT strain followed by the strain Wisconsin, however, neither strains showed large differences in *Cyp6g1* expression levels. In addition, the

susceptible strain 91-C was more tolerant to DDT than Canton-S, but the expression level of *Cyp6g1* was higher in Canton-S than 91-C. This is in contrast to data by Daborn et al. (2002) which has shown that in a range of field derived strains, the DDT resistant strains express 10-100 fold times more *Cyp6g1* mRNA when compared to a range field derived DDT susceptible strains (Daborn, Yen et al. 2002).

To show that the over transcription alone of *Cyp6g1* is sufficient for DDT resistance, Daborn et al. (2002) produced transgenic flies capable of over expressing *Cyp6g1*. The transgenic flies carried a copy of a construct containing *UAS-Cyp6g1*. These flies were crossed to another containing a heat-shock driver. This was used to drive the over expression of *Cyp6g1*. The levels of *Cyp6g1* transcript in the over expressing flies was shown to be about 100 times greater than in the control, non over expressing flies. When the over expression of *Cyp6g1* was driven using the heat-shock promoter, the percentage survival at a dose of 10 µg of DDT per treatment was increased from approximately 5-25% in the non heat-shocked flies to 80-100% in the heat-shocked flies. These experiments indicate that *Cyp6g1* over-transcription alone is sufficient for DDT resistance and that *Cyp6g1* over expression is necessary for DDT resistance.

DDT resistant strains over expressing *Cyp6g1* also show broad cross-resistance to the neonicotinoids, imidacloprid and nitenpyram and the insect growth regulator lufenuron (Daborn, Yen et al. 2002) as well as the neonicotinoid, acetamiprid and the organophosphate, malathion (Le Goff, Boundy et al. 2003). In addition, strains overexpressing *Cyp6g1* show an enhanced ability to metabolise the artificial substrate MROD (Jenkins, Dash et al. 2006). This broad cross resistance suggests that older classes of insecticide such as DDT can select for P450 mediated resistance which also confers resistance to new compounds with differing modes of action. Similar broad range cross resistance via a single resistance mechanism in a pest species would have important implications for the control of insects using insecticides.

2.1.2 Transposable elements and insecticide resistance

Sequencing of the resistance allele of two DDT resistant strains, Hikone-R and WC2 shows that both have an identical insertion in the 5' of *Cyp6g1*. The insertion shows homology to the terminal repeat of an *Accord* transposable element. All DDT resistant strains examined to date carry the insertion (Daborn, Yen et al. 2002). In many populations collected from different locations around the world, increased expression of *Cyp6g1* is correlated with the presence of this 491bp long terminal repeat (LTR) of an *Accord* retrotransposon 291bp upstream from the transcription start site of this gene (Daborn, Yen et al. 2002; Catania, Kauer et al. 2004). The exact mechanism whereby the *Accord* insertion up-regulates *Cyp6g1* transcription remains to be elucidated. Recent work by Daborn and coworkers using transgenic flies has shown that the *Accord* insertion contains tissue specific enhancers (personal communication). In *D. simulans* the insertion of a different transposon, a *Doc* element, is correlated with over-transcription of *Cyp6g1* (Schlenke and Begun 2004).

Transposable elements have been implicated in a number of cases of insecticide resistance. Transposons could cause resistance by either altering the metabolic enzymes that detoxify the insecticide or by altering the target of the insecticide (ffrench-Constant, Daborn et al. 2006). A long terminal repeat (LTR) of transposable element 17.6 inserted into *Cyp6a2* of *D. melanogaster* was thought to reduce the stability of the mRNA, thereby in the resistant fly, where the insertion is absent, the increased CYP6A2 would result in metabolic resistance (Waters, Zelhof et al. 1992). No correlation has been shown between the absence of the LTR and resistance to DDT (Delpuech, Aquadro et al. 1993). The insertion of a *Doc1420* element into the second exon of *CHKov1* in *D. melanogaster*, results in a functional, truncated protein (Aminetzach, Macpherson et al. 2005). The insertion of the *Doc1420* element confers low-level resistance to organophoshate insecticides over a short exposure period. Finally in the pest species *Heliothis virescens* the insertion of a retrotransposon in a putative Bt toxin receptor, truncates the protein and prevents the toxin binding. This resistance mutation has currently only been identified in laboratory selected *H. virescens*.

2.1.3 How may genes are required for resistance?

Whilst there is strong evidence that DDT resistance in both *D. melanogaster* and *D. simulans* is associated with over-transcription of *Cyp6g1*, prolonged laboratory selection with DDT also selects additional genes such as *Cyp12d1* (Brandt, Scharf et al. 2002) and *Cyp6a8* (Maitra, Dombrowski et al. 1996). Other studies have shown transgenic over expression of *Cyp6g1* to confer only low levels of DDT resistance and that recombinant strains derived from a highly DDT resistant strain, which lost over expression of *Cyp6g1*, were still resistant (Festucci-Buselli, Carvalho-Dias et al. 2005; Pedra, Festucci-Buselli et al. 2005). These results would suggest DDT resistance phenotype is more complex than the over-expression of *Cyp6g1*. Microarray analyses support the concept that several P450s can confer resistance DDT in *D. melanogaster* (Pedra et al., 2004). With intense laboratory selection of the strain WIS1 with DDT, a second P450 gene *Cyp12d1* was up-regulated (Le Goff, Boundy et al. 2003). When both of these genes were excluded by genetic recombination, further selection led to the up-regulation of a third P450 gene, *Cyp6a8*. Although a number of different P450 genes are capable of conferring resistance upon laboratory based selection, examination of recently collected field derived strains shows that a single gene, *Cyp6g1*, is always over-transcribed in DDT resistant *D. melanogaster* strains. *Cyp6g1* is therefore is a very important gene for study in insecticide resistance.

2.1.4 Aims of this chapter

The over-transcription of *Cyp6g1* in the DDT resistance allele is now a well-studied case of insecticide resistance. Several important questions relating to the mechanisms of over-transcription remain unanswered. In particular, although more *Cyp6g1* transcript can be detected in whole flies, is transcription up-regulated in all tissues or is over-transcription tissue specific? Furthermore, although there is strong evidence for the role of CYP6G1 in conferring insecticide resistance, what is the function of this P450 in wild type flies and what is the effect of knocking-down its expression. The aims of this chapter are to further investigate the role of *Cyp6g1* in insecticide resistance and to answer a number of important questions. First, is expression global within wild type flies or is

expression confined to specific tissues? Second, in flies over expressing *Cyp6g1*, do we see novel expression or is expression in the same tissues at higher levels. The UAS GAL4 expression system will be used to further characterise the role of *Cyp6g1* in DDT resistance by knocking down expression and examining the spatial expression. Finally, the ability of CYP6G1 to metabolise a number of compounds will be investigated.

2.2 MATERIALS AND METHODS

2.2.1 Fly maintenance

Stocks were maintained on a yeast and treacle diet (Appendix A1) at room temperature under constant light conditions. Stocks were routinely turned over every four weeks. Population sizes were maintained over approximately 200 flies.

2.2.2 Strains Used

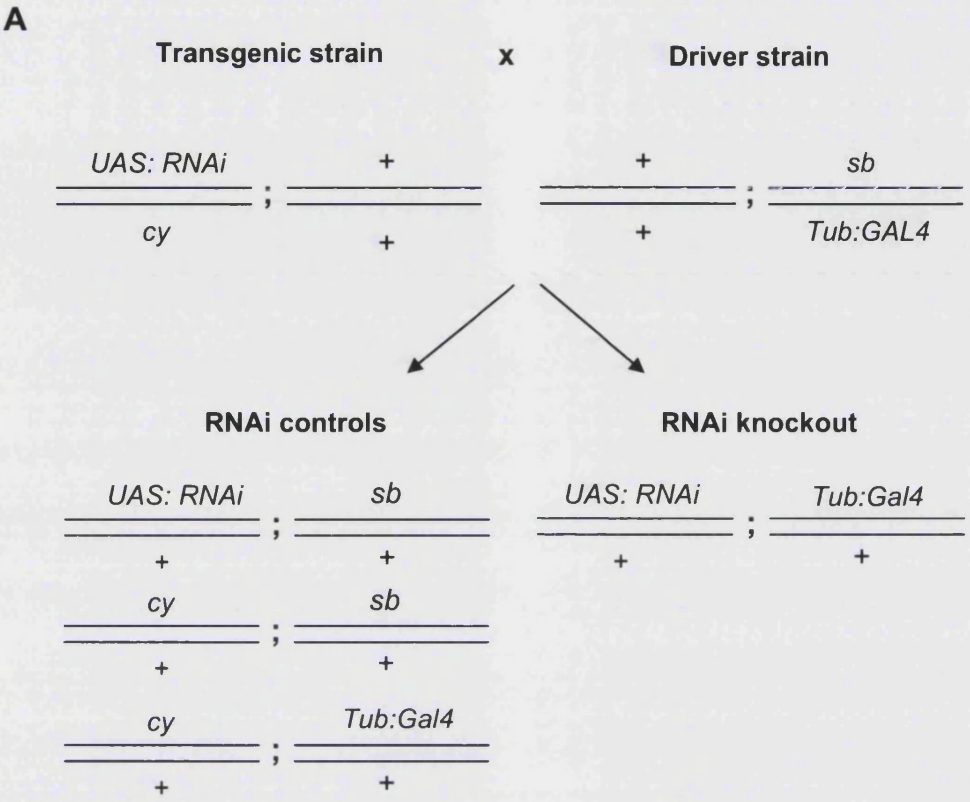
The laboratory standard strain Canton-S, obtained from the Bloomington Stock Centre was used as the wild type DDT susceptible strain. Hikone-R, originating from Japan was used as the DDT resistant strain. *UAS-6g1* strains were constructed by Phillip Daborn (University of Melbourne). The *UAS-RNAi* strain was obtained from the Nig-Fly project (http://shigen.lab.nig.ac.jp/fly/nigfly/). This strain contains a construct expressing short hairpin RNA of *Cyp6g1* under the control of the UAS promoter. GAL4 driver strains were obtained from the Bloomington Stock Centre.

2.2.3 Knockout of *Cyp6g1*

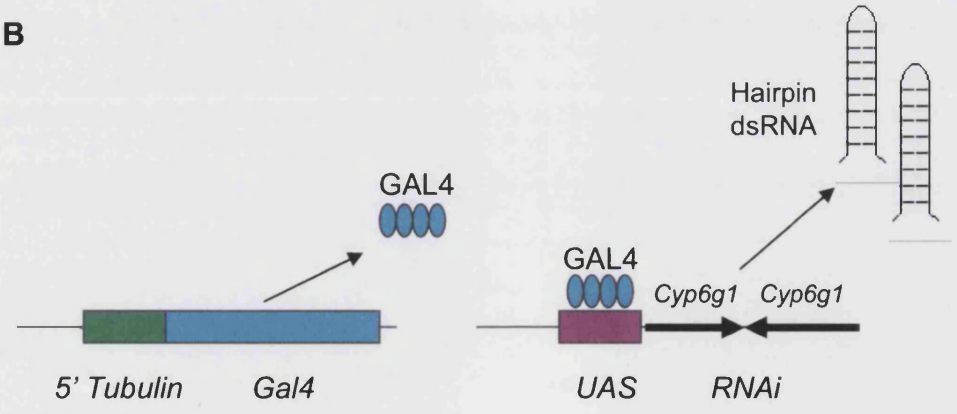
2.2.3.1 RNAi using UAS-hairpin

Virgin females of the *UAS-RNAi* strain were crossed to males of the driver strain *tubulin-Gal4/TM3* (Bloomington stock number 5138). The *tubulin-Gal4* driver line was used to drive expression of the short hairpin RNA (shRNA) throughout the lifespan of the fly. Control flies (*sb/UAS-RNAi*), not expressing the shRNA were distinguishable by the presence of the stubble marker (figure 2.1). Flies without the stubble marker (*Gal4/UAS-RNAi*) contain both the *tubulin-Gal4* and the *UAS-RNAi* construct and had therefore express the shRNAs. This is intended to knock down expression of *Cyp6g1*.

Figure 2.1. RNAi using the GAL4/UAS expression system



*Control flies (*sb/UAS-RNAi, sb/cy, Gal4/cy*) are distinguishable by the presence of the stubble and/or curly wing marker. Flies without the stubble marker (*GAL4/UAS-RNAi*) contain both the* Tubulin-Gal4 *and the* UAS-RNAi *construct*



The tubulin promotor results in the ubiquitous expression of GAL4. GAL4 binds to UAS *in order to activate the transcription of the inverted repeat of* Cyp6g1 *resulting in the production of the shRNAs responsible for the RNAi phenotype.*

2.2.3.2 Bioassays

Bioassays were performed using 23 ml scintillation vials (Wheaton). DDT was prepared in acetone at the following concentrations: 2.5, 5.0, 10.0, 20.0 µg/ml. 200 µl of the DDT in acetone was added to each vial and the vial rolled on its side to evenly distribute the solution until all the acetone had evaporated. Control vials with just acetone were also rolled. Vials were left for 2 h to completely dry. For each strain five vials of each DDT dose were prepared and five vials of solely acetone. Female flies aged 3-5 days were collected and sorted for the presence of the stubble marker. 20 flies were added to each vial and the vial was bunged with a piece of cotton wool soaked in 5% (w/v) sucrose solution. Vials were stored in an incubator for 24 hours and then each vial was scored for the number of dead flies. Paralysed flies were counted as dead. Data were analysed in Excel (Microsoft) and MINITAB 12.0 using a one way T Test at the 5% level.

2.2.4 Quantifying *Cyp6g1* expression

2.2.4.1 Quantitative real time PCR

A quantitative real time PCR (Q RTPCR) was performed to quantify *Cyp6g1* in the strains Hikone-R (RR), Canton-S (SS), the RNAi knockout (*Gal4/UAS-RNAi*) and the RNAi control flies (*sb/UAS-RNAi*). Flies were collected at 5 days old and stored until needed at -80ºC. For the RNA extraction, 10 flies were used per sample. Three repeats were performed per strain. RNA was extracted using the RNeasy Mini (Qiagen) protocol following manufacturers instructions. 10 µl of the RNA was used to make cDNA. CDNA was prepared using the Superscript II cDNA Synthesis System (Invitrogen) kit following manufacturers instructions. To test for the presence of DNA contamination, a control with no Superscript II enzyme was run in parallel and all of the samples run in a PCR assay to confirm the presence of DNA in the Superscript II positive reactions and that no DNA was present in the no Superscript II control. PCR mixtures using 1 µl of the Superscript reaction in 17 µl 1.5x Reddymix (Abgene) and 0.5 µl each of the primers. The primers used to check for contamination were RP49F-ATCCGCCCAGCATACAG, RP49R-TCCGACCAGGTTACAAGAA, 6G1F-

CGGCTGAAGGACGAGGCTGTG and 6G1R-GCTATGCTGTCCGTGGAGAACTGA. PCR conditions were 95°C for 2 min and 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final extension step of 72°C for 10 min. 5 µl of PCR product was run on a 0.8% agarose gel stained with ethidium bromide and viewed under a gel viewer (BioRAD). DNA from one PCR reaction of each was purified using a PCR Purify Kit (Qiagen) and quantified using a spectrophotometer. The purified DNA was diluted to make standards for the QPCR.

QPCR mixtures of 20 µl, containing 1 µl of DNA from single flies, 1 µl of each 10pmol/µl primer and 18 µl of SYBR green QPCR mix (Finnzymes) were used for the QPCR reaction. The primers used were those described above for detecting DNA contamination. The PCR conditions were as follows: 95°C for 15 min to activate the DNA polymerase, followed by 30 cycles of 94°C for 30 sec for denaturation, 55°C for 30 sec annealing stage and 72°C for 30 sec for the extension phase. The reactions were run in an MJ Research Opticon machine and data were analysed in Opticon monitor software 3.0, Microsoft Excel and MINITAB.

2.2.4.2 Microsome preparation

Drosophila flies of strains Hikone-R, Canton-S, *Gal4/UAS-RNAi* and *sb/UAS-RNAi* were collected at 7 days old and snap frozen in liquid nitrogen and stored at -80°C. All work was carried out on ice. Fly heads were removed by sieving (850 µm aperture, Fisher). 2 ml flies were crushed in 5 ml of buffer (100 mM phosphate buffer pH 7.2 (K2HPO4/KH2PO4), 1 mM EDTA, 0.1 mM DTT, 0.4 mM PMSF) with a hand homogeniser. Homogenate was filtered on muslin, filtered buffer was centrifuged at 10,000 g for 15 min at 4°C. The resulting supernatant was centrifuged at 100,000 g for 1¼ h at 4°C. The pellet was resuspended in 1 ml 100 mM phosphate buffer containing 20% glycerol. A Bradford assay was then carried out on these samples to find the total protein concentration. A standard curve was generated using known amounts of BSA (New England Biosciences). 20 µl sample, 80 µl water and 900 µl Bradford reagent (Sigma) were combined in a 1 ml polyacrylamide curvette and left to stand for 10 min.

The samples were then inverted once to mix and the absorbance at 595 nm read using a spectrophotometer. A Microsoft Excel worksheet was then used to calculate the sample concentration based on the standard curve. The samples were diluted to the desired concentration based on this result.

2.2.4.3 Methyl Ether Resorufin (MROD) metabolism

Cyp6g1 activity was quantified with the model substrate 7-methoxy-3H-phenoxazin-3-one (Methyl Ether Resorufin, Sigma). Initially the reaction was prepared with 0.25 mM MROD in ethanol, 100 mM phosphate buffer pH 7.8 (Na_2HPO_4 and NaH_2PO_4), 2.5 mM NADPH and 30 µl of homogenised fly in 100 mM phosphate buffer. Fluorescence was measured using a Tecan Ultra Fluorescence detector. Homogenised fly preparations did not show any activity towards MROD so 200 µg microsomes were used. Additional reactions containing 30 µl homogenised Malpighian tubules and midgut from Canton-S, Hikone-R, *sb/UAS-RNAi* and *Gal4/UAS-RNAi* in phosphate buffer were also prepared. Data were analysed in Microsoft Excel.

2.2.4.4 Imidacloprid metabolism

In an attempt to show *Cyp6g1* is involved in insecticide metabolism, in vitro incubations of microsomes and the insecticide imidacloprid were prepared following a protocol developed by Karine Bourcier, Pfizer Animal Health. Imidacloprid was run through a mass spectrophotometer (Perkin Elmer Sciex, API 2000) to obtain a profile for the compound. Incubations contained 50 mM Tris HCl, 20 mM $MgCl_2$ $6H_20$, 0.1 mM NADPH (Sigma), 2 mM Isocitric acid (Sigma), 20 mM Isocitric dehydrogenase (type IV, Sigma). The reagents were incubated in a shaking waterbath at 37°C for 5 minutes before the 35µl 20 µg/ml imidacloprid in methanol was added. At 0, 5, 10, 20, 40, 60 minutes post Imidacloprid addition a 100 µl sample was taken and put onto 200 µl Methanol on ice to stop the reaction. Samples were spun at 4000 rpm to pellet protein residues and 100 µl of the sample was run through an HPLC analysis (Perkin Elmer series 200 autosampler and series 200 micro LC pump). Data were

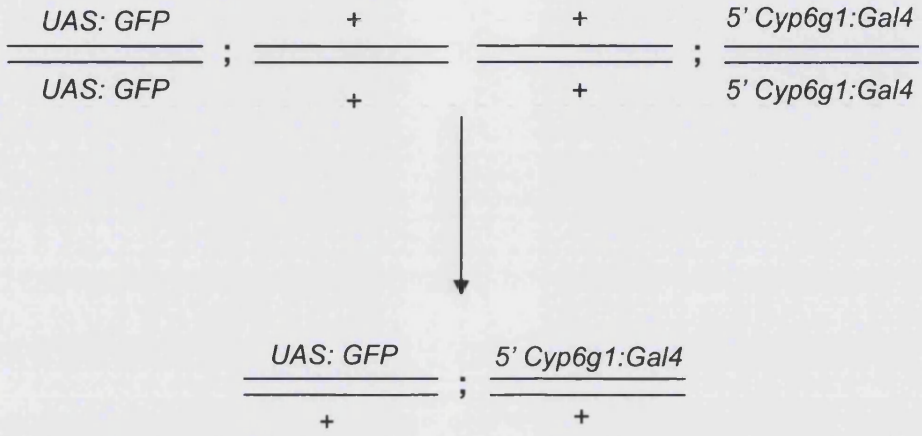
collected in Analyst Software 1.3.1 (Applied Biosystems) and analysed in Microsoft Excel. As the initial incubations did not show the expected drop in imidacloprid concentration further repeats of the incubations were attempted at 32°C and 25°C.

2.2.5 Green Fluorescent Protein expression using UAS/GAL4 expression system

The UAS/GAL4 expression system was also used to study the pattern of expression of *Cyp6g1*. Constructs were made by Phillip Daborn, University of Melbourne. The upstream region of *Cyp6g1* consisting of 1608 bp to the start methionine, was amplified by PCR from Hikone-R using Expand High Fidelity PCR system (Roche) and the primers MBF1 (ATT TGA TCC CGT CAT TTC GCC) and 5p2R (TTT GGG GAT GTC GAT GTA ATG). The equivalent 1200 bp and 1197 bp fragments were amplified from Canton-S. One of these constructs has the resistant 5' regulatory sequence encompassing the *Accord* fragment, the other has the Canton-S susceptible 5' regulatory sequence. These strains were crossed to the driver strain *UAS-GFP,* which expresses Green Fluorescent Protein (GFP) when the Upstream Activation Sequence (UAS) is induced (figure 2.2). The adult flies were collected at 5 days old and dissected in Ringers dissecting medium (appendix A3). Preparations were fixed using Prolong Antifade Kit (Invitrogen,) under a dissecting microscope (Zeiss). Slides were viewed under the confocal microscope (Zeiss) and images viewed with the Zeiss LSM meta browser software.

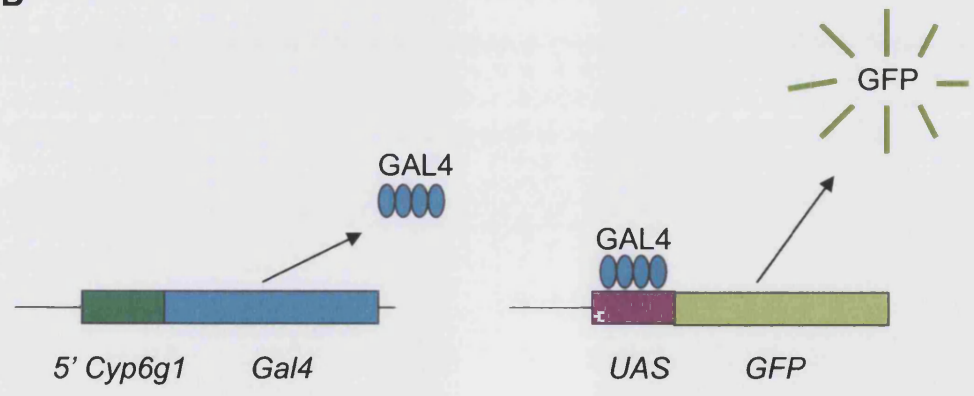
Figure 2.2. Expression of GFP using the GAL4/UAS expression system in the resistant and susceptible

A



Flies express GFP regulated by the 5' region of either Canton-S i.e. the susceptible pattern of expression or Hikone-R (with the Accord *insertion) i.e. the resistant pattern of expression*

B



The Cyp6g1 *promoter drives the expression of GAL4. GAL4 binds to* UAS *and activates the transcription of GFP. Fly strains with the 5' promoter region of* Cyp6g1 *from both resistant and susceptible flies were created.*

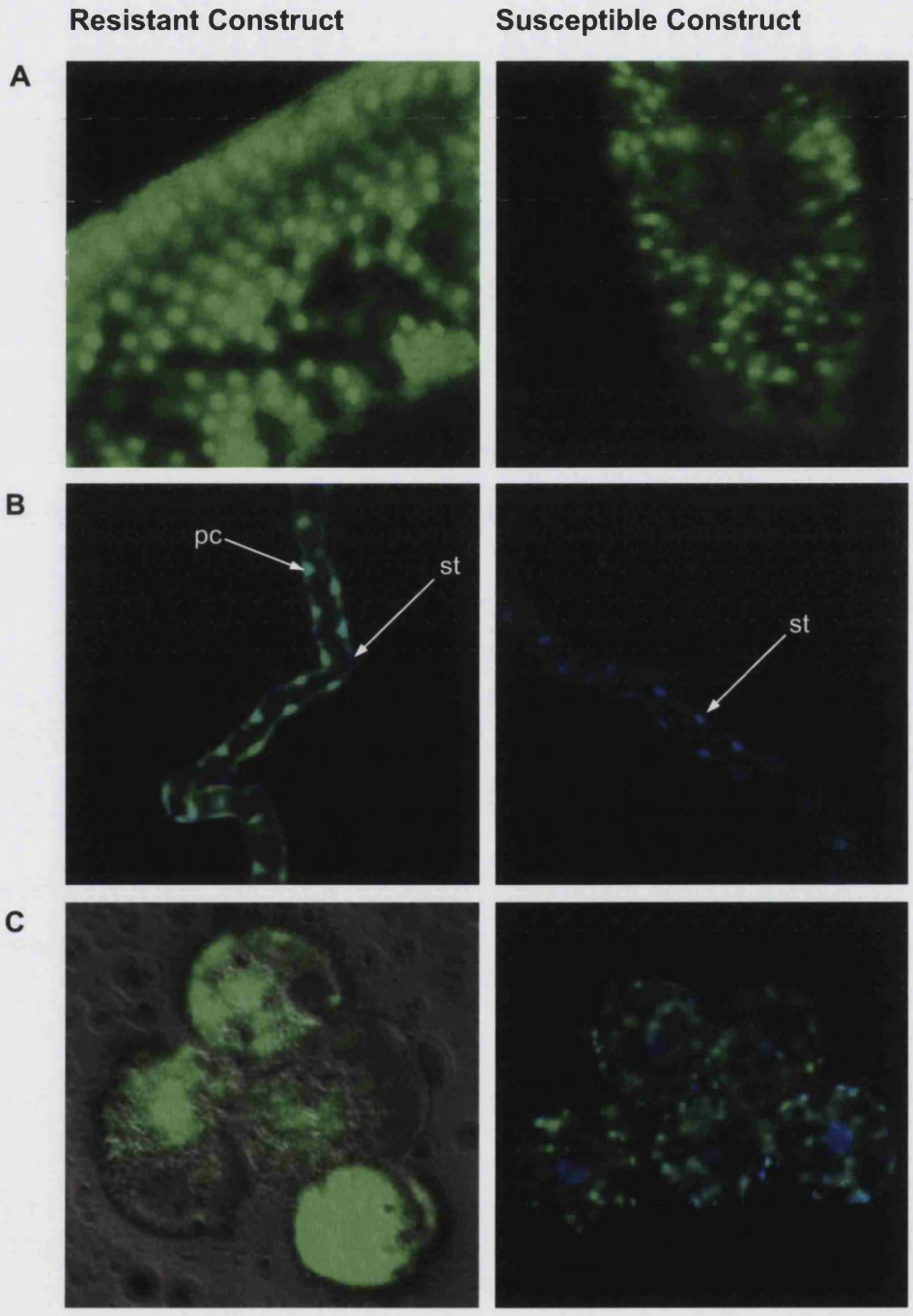
2.3 RESULTS AND DISCUSSION

2.3.1 Spatial expression of *Cyp6g1*

To determine the spatial expression of *Cyp6g1* in insecticide resistant and susceptible flies, the Gal4/UAS expression system was used to express GFP regulated by the 5' of *Cyp6g1* allowing a comparison of expression in flies with the resistant and susceptible regulatory sequences. The tissues showing greatest differences between susceptible and resistant constructs in adult flies were the midgut, Malpighian tubules and fat body (figure 2.3). These images were taken with identical microscope settings allowing the direct comparison of the expression in the tissues of the resistant and susceptible GFP expressing *Drosophila.* The UAS GFP strain used expresses nuclear GFP resulting in the nuclear expression patterns. The findings were consistent with data from Chung et al. (in Press) that looked at expression in 3rd instar wandering larvae using the same constructs.

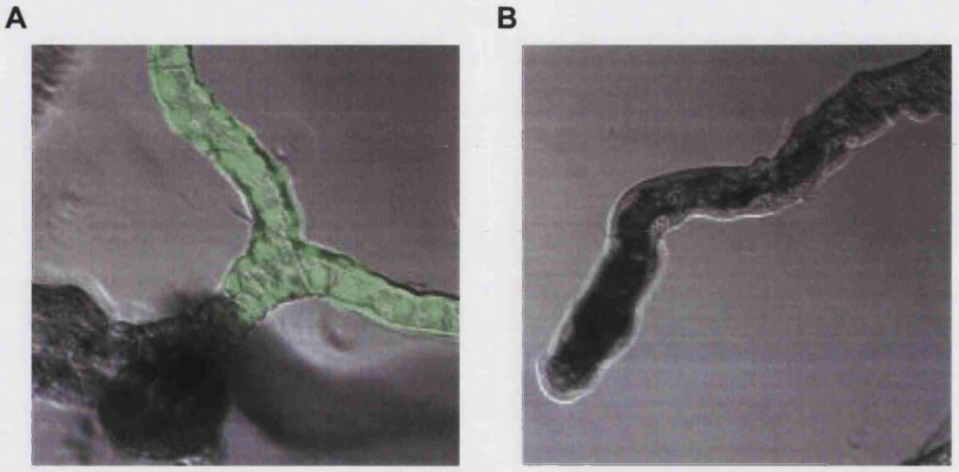
The main segment of the Malpighian tubule shows strong expression of GFP. DAPI staining shows the co-localisation of GFP and DAPI in the nuclei of the primary cells however in the smaller stellate cells only DAPI staining can be seen (figure 2.3). This indicates expression is solely in the primary cells of the main segment (figure 2.3) suggesting that the primary cells play a specialized role in such detoxification. The GFP expression in the Malpighian tubules does not extend to the initial segment of the Malpighian tubule (figure 2.4) or beyond the junction with the ureter. The main segment of the Malpighian tubule is responsible for pumping ions and water from the basolateral blood side to the apical side. A detailed microarray study of gene expression in adult *Drosophila* Malpighian tubule (Wang, Kean et al. 2004) revealed substantial up-regulation of several members of the cytochrome P450 and glutathione-S-transferases. The group found *Cyp6g1* to be nearly 10 fold enriched in the Malpighian tubule when compared with the rest of the fly (table 2.1).

Figure 2.3. GFP expression driven by GAL4/UAS expression system using driver strains with 5' Hikone-R (resistant) and 5' Canton-S (susceptible) promotor regions. DAPI staining is shown in blue.



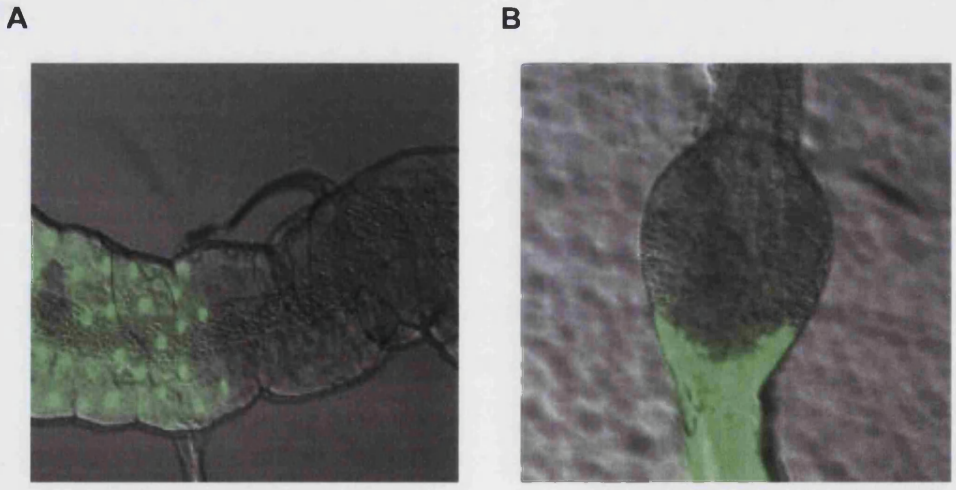
Confocal images comparing resistant and susceptible constructs A. a section of the midgut B. part of the main segment of Malpighian tubule showing the stellate cells (st) and primary cells (pc) and C. the fat body

Figure 2.4. GFP in 5' Hikone-R (resistant) expression patterns in the Malpighian tubule.



Confocal images showing A. the junction of the ureter and Malpighian tubule and B. the initial segment of the Malpighian tubule.

Figure 2.5. GFP in 5' Hikone-R (resistant) expression patterns in the digestive tract.



Confocal images showing A. the junction between the midgut and hindgut and B. the proventriculus.

Drosophila gene	Enrichment in tubule
Cytochrome P450s	
Cyp6a18	25.5
Cyp4p3	23.5
Cyp6a8	11.1
Cyp6a21	9.6
Cyp6g1	9.4
Cyp310a1	8.9
Cyp4d2	8.3
Cyp6a2	7.9
Glutathione-S-transferases	
CG17522	39.5
CG17527	7.3
CG17531	6.7
CG17524	5.5
CG17531	4
BcDNA:GH04753	3.9
CG6776	3.9

Table 2.1. *Detoxification genes that are strongly up-regulated in* Drosophila *Malpighian tubules when compared with whole flies. From (Wang et al., 2004).*

GFP expression in the midgut is higher in the resistant construct flies than the susceptible (figure 2.3). Expression appears to be in the majority of the goblet cells of the midgut. GFP expression shows a clear distinction between the mid and hindguts (figure 2.5). Expression extends to the proventriculus in both resistant and susceptible flies. Young (1-2 day old) *D. melanogaster* have pericuticular fat bodies, noticeable in greater abundance compared with older flies. These fat deposits show strong GFP expression in the resistant construct flies but much lower GFP expression in the susceptible construct flies. The presence of CYP6G1 in the fat body of the flies would be expected to be beneficial in the detoxification of insecticides, as this would provide a second barrier to DDT after the cuticle. The insect fat body stores carbohydrates, lipids and proteins that are used through energy demand modulated by neurohormones combining important roles in the insect metabolism resembling those of the vertebrates liver and adipose tissue (van der Horst et al., 1997). The expression in the Malpighian tubules is consistent with the observation by Wang et al. that CYP6G1 is enriched in the tubules compared with the rest of the fly (Wang, Kean et al. 2004). When the over expression of *Cyp6g1* is specifically

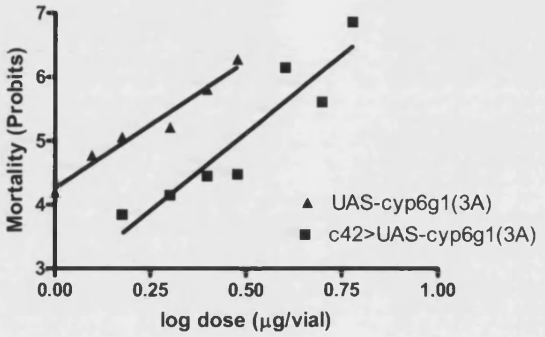
driven by the c42 GAL4 driver strain that drives expression specifically in tubule principal cells, the sensitivity to DDT decreases in these flies (figure 2.6). In addition when the knock down of the expression is driven specifically in the principal cells, the sensitivity to DDT shows a slight increase (data not shown).

Cyp6a1 in *M. domestica*, expression has been identified in the digestive system and fat bodies in house fly larva (Carifio et al., 1994). The cytochrome P450 1pr has been identified in the fat bodies, proximal intestine, reproductive system and Malpighian tubules in adult house fly (Scott and Lee, 1993; Lee and Scott, 1992). In a laboratory selected strain of *D. melanogaster* that is resistant to DDT, *Cyp6a2* is also constitutively overexpressed in the midgut, the pericuticular fat bodies and the Malpighian tubules (Brun, Cuany et al. 1996). Basal expression of *Cyp6a2* in Canton-S flies takes place in the digestive system where the expression level is very low. Phenobarbital treatment promotes significant expression of *Cyp6a2* in the digestive system, the fat bodies and the Malpighian tubules.

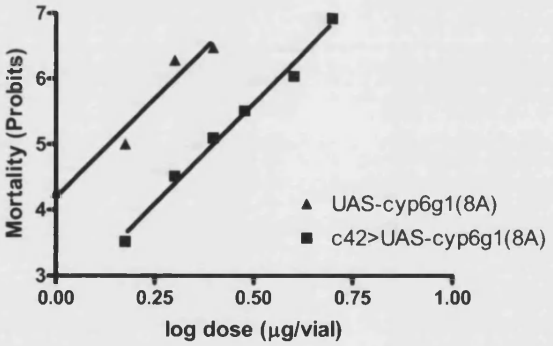
The finding of Cytochrome P450 expression in the Malpighian tubules is logical as these organs are responsible for the excretion of foreign compounds from the fly. The expression in the digestive tract is also logical given the main route of entry for the insecticide is likely to be through ingestion. Finally the expression in the fat body is consistent with providing a secondary barrier after the cuticle by which to prevent the spread of the insecticide within the insect body. The locations of the expression of *Cyp6g1* and other Cytochrome P450s in resistant insects match the tissues in contact with xenobiotics when they enter into the flies (digestive system and pericuticular fat bodies) or when they are excreted (Malpighian tubules).

Figure 2.6. Over expression of *Cyp6g1* in tubule principal cells driven by the UAS GAL4 expression system compared with a strain showing no over expression

A



B



(A) Dose-response curve for Cyp6g1 Malpighian tubule specific over-expression strain Cyp6g1-3A compared with its UAS parent line. (B) Dose-response curve for Cyp6g1 Malpighian tubule specific over expression mutant of Cyp6g1-8A compared with its UAS parent line.

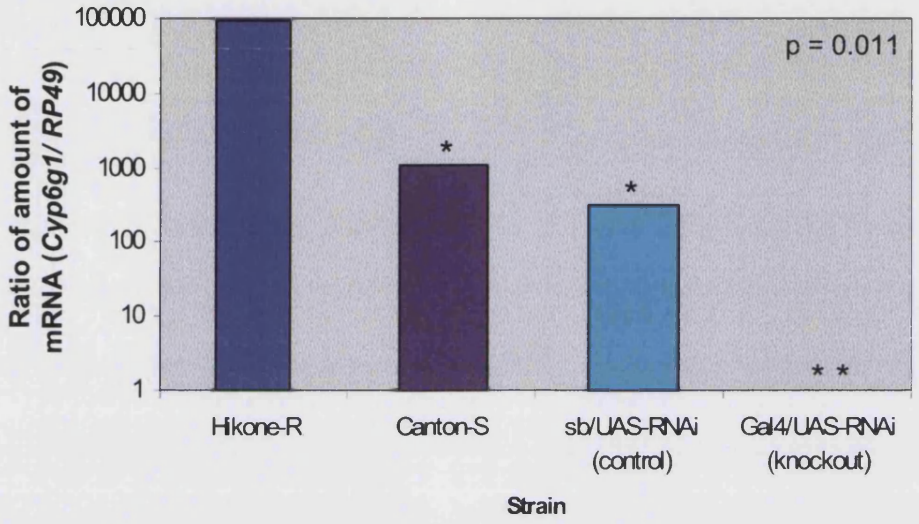
2.3.2 Quantifying *Cyp6g1* expression

2.3.2.1 Knock down of *Cyp6g1*

Quantitative real time PCR was used to measure the amount of *Cyp6g1* mRNA transcript in the strains Hikone-R, Canton-S and *Gal4/UAS-RNAi* (RNAi strain) and *sb/UAS-RNAi* (RNAi control). The results show the relative amount of *Cyp6g1* transcript to be 100 fold higher in Hikone-R compared with Canton-S (figure 2.7). This concurs with data collected by Phillip Daborn (Daborn, Yen et al. 2002). The amount of *Cyp6g1* transcript in the RNAi control is lower than Canton-S reflecting the natural variation of *Cyp6g1* expression in different strains. There was no detectable *Cyp6g1* mRNA in the RNAi knockout indicating the knockdown of *Cyp6g1* mRNA was extremely effective. Ideally the amount of the CYP6G1 protein produced by the flies would also be measured. Very small amounts of mRNA, undetected by the quantitative real-time PCR would result in the production of the protein. A western blot using the *Cyp6g1* antibody to detect CYP6G1 in whole flies preparations would confirm that no CYP6G1 protein was present in the flies.

The RNAi strain (*Gal4/UAS-RNAi*) is viable and shows no phenotypic defects compared with the control, *sb/UAS-RNAi* indicating *Cyp6g1* does not have a vital function in *D. melanogaster*. This is in contrast to results from constructs made Phillip Daborn, which appear to be lethal (personal communication). In order to ensure that the position of the insertion does not affect the flies, ideally several different strains generated by separate insertions of the RNAi construct would have been tested. The lack of any obvious phenotype in the RNAi flies gives no clue as to what the normal function of CYP6G1 may be.

Figure 2.7. Ratio of the amount of *Cyp6g1/RP49* mRNA in Hikone-R, Canton-S and the RNAi strains measured using Q RT-PCR



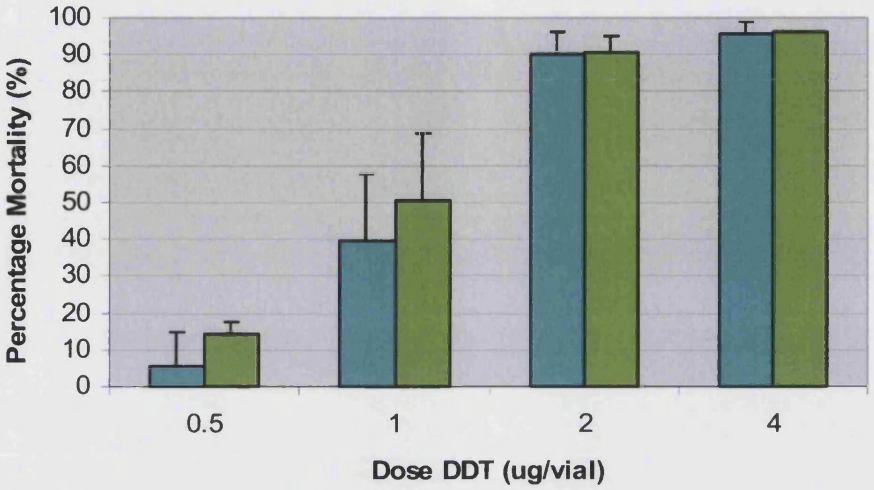
*The amount of mRNA of each strain is plotted as a ratio of Cyp6g1 to the ribosomal protein RP49. * and * * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons) and the p value is given.*

2.3.2.2 DDT bioassays

Bioassays of the RNAi strain and the RNAi control indicate there is a slight increase in the mortality of the RNAi strain at 0.5 and 1 ug DDT per vial compared with the control (figure 2.8). This increase is not significant ($T = 0.62$, $p = 0.54$) however the trend indicates that knockdown of *Cyp6g1* results in a reduction in survival at low doses of DDT exposure. The dose response graphs show only a very slight difference between the strains (figure 2.9). The control strain shows levels of *Cyp6g1* transcript similar to the DDT susceptible strain Canton-S so the RNAi control shows low, wild type levels of *Cyp6g1* expression. At these low doses of DDT, other detoxification mechanisms may be acting. Therefore DDT metabolism in susceptible flies is normally at such a low level that knock-down produces no significant effects on insecticide mortality.

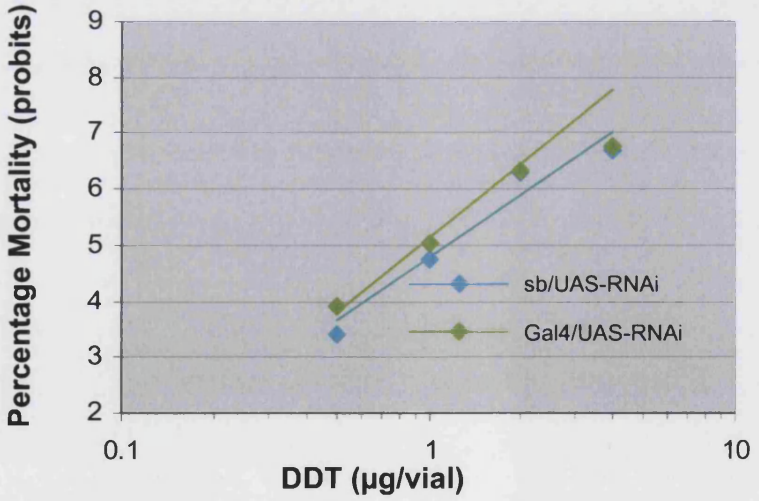
Cyp6g1 is inducible by the compound phenobarbital. The expression of *Cyp6a2* in Canton-S has been shown to be under the control of phenobarbital (Brun, Cuany et al. 1996). When adult flies were treated with phenobarbital, a 15-fold increase in the transcription of *Cyp6a2* was described in the midgut, the fat bodies and the Malpighian tubules. In a DDT selected strain that is resistant to the insecticide, this gene is constitutively over expressed in the same tissues. By exposing the RNAi and RNAi control flies to phenobarbital it would be expected that the difference in CYP6G1 would be greatly increased between the strains and thus the differences in the response of the strains to DDT would be greater. This would be a simple method of confirming that knock out of *Cyp6g1* reduces susceptibility to DDT. The method would not eliminate the possibility that the phenobarbital is inducing other Cytochrome P450s that can affect resistance. Alternatively by crossing the *UAS-RNAi* construct into an over-expressing strain, e.g. Hikone-R the knock down would be in a strain expressing higher levels of CYP6G1 and would be expected to have a greater effect. This method would be preferable as the chemical compounds used to induce P450 expression may result in a synergistic or antagonistic effect on DDT resistance.

Figure 2.8. Bioassay with DDT of RNAi knockout (*Gal4/UAS:RNAi*) and RNAi control (*sb/UAS:RNAi*).



The mean percentage mortality for each strain is plotted for a number of doses of DDT. The knockout strain is indicated in green, the control strain in blue.

Figure 2.9. Dosage mortality curves for DDT of RNAi knockout (*Gal4/UAS:RNAi*) and RNAi control (*sb/UAS:RNAi*).



2.3.2.3 MROD assay

MROD is a water-soluble molecule that can be metabolised into the fluorescent O-dealkylated substrate, resorufin. MROD metabolism has previously been shown to correspond to P450 activity (Jenkins, Dash et al. 2006). The combined midgut and Malpighian tubules of Hikone-R, Canton-S, RNAi and RNAi control strains were dissected and the tissues crushed in a phosphate buffer. These preparations were added to the MROD assay and the production of the flurophore, resorufin was quantified. The MROD assay indicates that the P450 activity is highest in the Malpighian tubules of resistant Hikone-R flies and the *Sb/UAS-RNAi* genotype, which acts as a control for the RNAi flies (figure 2.10). This is in comparison with the standard susceptible strain Canton-S and the RNAi flies (*Gal4/UAS-RNAi*), which show limited MROD metabolism as consistent with the hairpin RNAi construct totally suppressing *Cyp6g1* transcription.

The ability of microsomes prepared from Hikone-R to metabolise MROD was much greater than Canton-S (figure 2.11). This is consistent with the results obtained from isolated midgut and Malpighian tubule preparations. The activity of the ability of the RNAi flies to metabolise the artificial substrate MROD was apparently eliminated (figure 2.11). The *Sb/UAS-RNAi* genotype, which acts as a control for the RNAi flies, shows only low levels of activity towards MROD. This is surprising as Q-RT-PCR results indicate *Cyp6g1* mRNA transcript to be not significantly higher in Canon-S. This result indicates that Canton-S microsomes contain other compounds, possibly Cytochromes P450 that metabolise MROD, which are not present in the RNAi strain. RNAi control flies still show a low rate of metabolism of the artificial substrate MROD however, which can in turn be abolished by RNAi. This suggests that wild type flies do contain enough CYP6G1 to perform active metabolism of xenobiotics but the DDT bioassays indicate that the removal of the enzyme does not seem to significantly increase the overall susceptibility of susceptible flies.

Figure 2.10 Methyl Ether Resorufin (MROD) metabolism by preparations of Malpighian tubules and midguts from Canton-S, Hikone-R, RNAi knockout (*Gal4/UAS:RNAi*) and RNAi control (*sb/UAS:RNAi*).

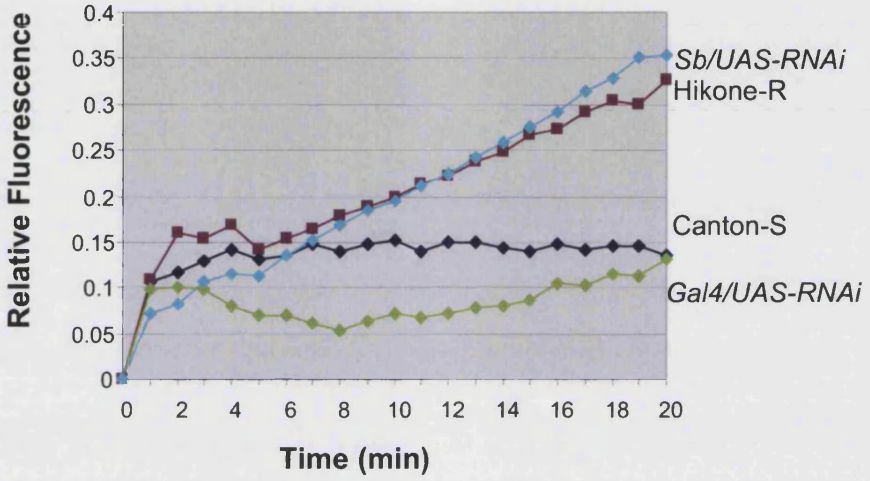
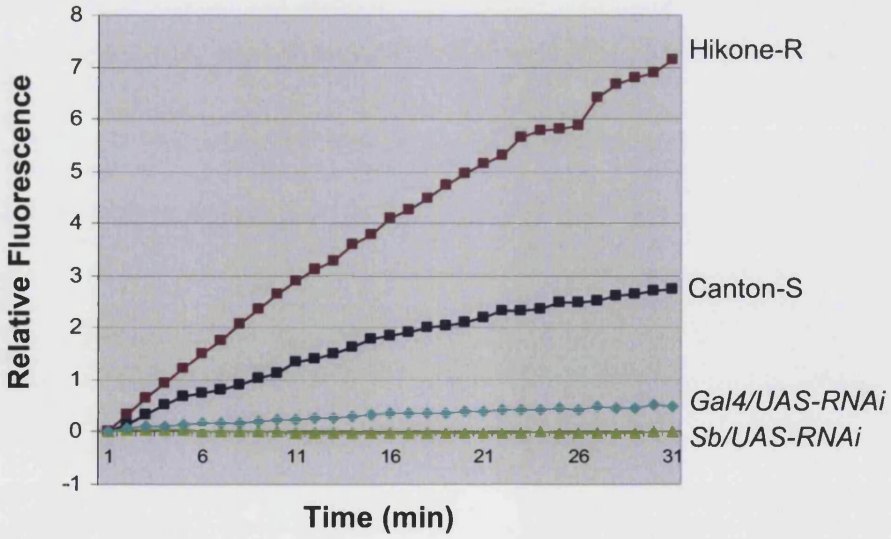


Figure 2.11 Methyl Ether Resorufin (MROD) metabolism by microsomes prepared from whole flies of the strains Canton-S, Hikone-R, RNAi knockout (Gal4/UAS:RNAi) and RNAi control (sb/UAS:RNAi).

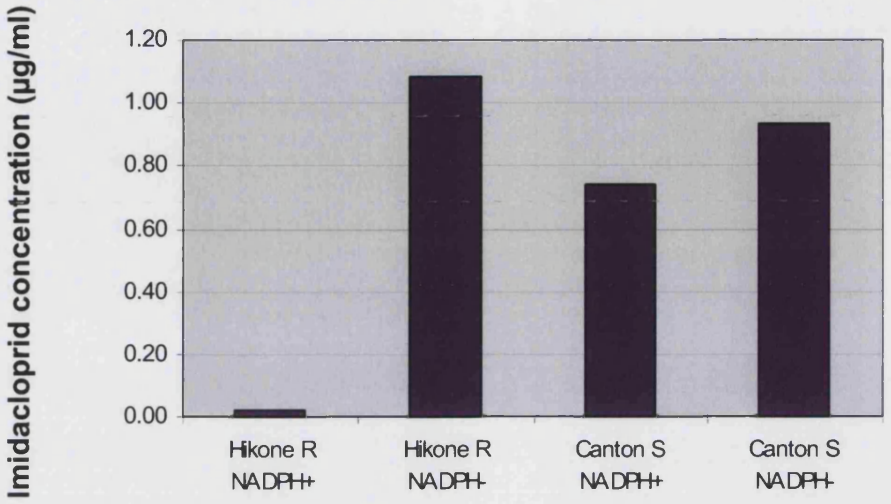


2.3.2.4 Imidacloprid metabolism

To try to show *Cyp6g1* can metabolise imidacloprid, in vitro incubations of microsomes with imidacloprid were prepared and the reaction stopped at a number of time points before the concentration of imidacloprid remaining was determined by LC-MS. As the initial incubations at 37ºC did not show a drop in the imidacloprid concentration at 60 minutes, further repeats of the incubations were attempted at 32ºC and 25ºC. These temperatures are likely to be more representative of the temperatures at which the enzymes function in the fly. The initial incubation at 25ºC gave a promising result (figure 2.12) with a lower concentration of imidacloprid in the sample incubated with NADPH compared with those incubated without NADPH. The reduction was greater in Hikone-R compared with Canton-S, consistent with the higher rate of MROD metabolism by the Hikone-R microsomes. When the incubations were repeated a further 4 times under the same conditions, the concentration of imidacloprid was not reduced in any of the samples without NADPH when compared with the samples with NADPH. Repeating the procedure with fresh microsomes resulted in no decrease in the imidacloprid concentration after the 60-minute incubation in any of the samples. Further attempts at the assay involved altering the starting concentration of imidacloprid and increasing the starting microsome concentration. None of the alterations resulted in a drop in the concentration of imidacloprid over a 60-minute period.

The results suggest that the data from the initial incubation were an anomaly, since it was not possible to repeat this result. It is possible the imidacloprid leaked from the system or that the initial high concentrations of imidacloprid were due to inadequate mixing of the sample. To check the microsome activity, the microsomes were tested in the MROD assay and were shown to metabolise MROD as expected. The MROD metabolism was at a constant rate up to 60 minutes after the incubations were started so it would be expected that the degradation of the microsomes was not a problem during the imidacloprid metabolism. The reactions may have been lacking a cofactor necessary for the reaction. Examples of possible cofactors necessary for the reaction could include ATP or sugars. Alternatively the reactions may contain an inhibitor, preventing the metabolism of imidacloprid.

Figure 2.12. Imidacloprid metabolism by microsomes from Canton-S and Hikone-R, with and without NADPH.



The concentration of imidacloprid measured by liquid-chromatography/mass spectrometry after a 60 minute incubation time with and without the addition of NADPH is plotted for Hikone-R and Canton-S.

An alternative method that may be used to show that microsome preparations can metabolise imidacloprid would be to measure the production of the imidacloprid metabolite. This method is preferable in that the concentration of imidacloprid in the reaction may drop but not die to the metabolism of the compound by the microsomes. The imidacloprid concentration may drop due to imidacloprid leaking from the system. The measure of the metabolite confirms the ability of the microsomes to metabolise the compound.

2.3.3 Conclusions

The use of the UAS GAL4 expression system in *Drosophila* is a useful tool for studying the role of *Cyp6g1* in DDT resistance. By driving GFP expression with the regulatory sequence of *Cyp6g1*, the spatial expression of *Cyp6g1* has been identified. The pattern of expression of GFP by the susceptible 5' construct suggests that CYP6G1 is found in the midgut, Malpighian tubules and fat body of adult flies. The pattern of over-expression of GFP by the resistant 5' construct suggests that CYP6G1 is expressed in the same subset of tissues but in greater quantities, consistent with the *Accord* element simply increasing existing promoter activity.

In addition the UAS Gal4 expression system has been used to knock down *Cyp6g1* expression. Knock down has been shown to reduce DDT resistance but not significantly. This is probably because DDT metabolism in susceptible flies is normally at such as low level that its knock-down produces no significant effects on insecticide mortality. The susceptible flies still show an appreciable rate of metabolism of the artificial substrate MROD, which can in turn be abolished by RNAi. This suggests that wild type flies produce enough CYP6G1 to perform active metabolism of xenobiotics but that removal of this enzyme does not seem to significantly increase the overall susceptibility of susceptible flies. These results support existing evidence for role of *Cyp6g1* in DDT resistance.

Further questions remain unanswered. First, the exact mechanism whereby the *Accord* element up-regulates transcription, in an apparently tissue specific fashion, needs to be resolved. Chung et al (in press) cloned the 491 bp *Accord*

insertion and used this to drive nuclear GFP expression. The expression patterns match those seen with the 1608bp fragment driving nuclear GFP expression in the Gal4 driver strain used in this chapter. This suggests that the *Accord* LTR carries its own tissue specific enhancers that are capable of expressing *Cyp6g1* in the midgut, Malpighian tubules and fat body. In addition, current work by Daborn et al. has found that in Hikone-R, *Cyp6g1* is duplicated along with the *Accord* insertion, contributing to high level *Cyp6g1* expression and the associated DDT resistance (personal communication).

Second, as the RNAi flies show no obvious phenotypes, we do not have any indication as to the normal function of CYP6G1. Since *Cyp6g1* is expressed in the Malpighian tubules and shows broad cross resistance to a number of compounds with varying modes of action, it seems likely that the wild type function of *Cyp6g1* may be in the detoxification of toxic compounds found in the wild. The presence of the *Accord* insertion in wild populations at frequencies approaching fixation despite the ban of DDT use indicates that there may be little or no cost to carrying the *Accord* element, and hence little or no cost associated with *Cyp6g1* overexpression. The following chapters will explore the possibility that the *Accord* confers a fitness benefit and may play a role in reproduction.

CHAPTER 3

Fitness of *Accord* element in the absence of DDT selection

3.1 INTRODUCTION

3.1.1 Costs of resistance

Predicting the rate at which resistance may evolve is important in the development of strategies to prevent or slow the evolution of insecticide resistance (Roush and McKenzie 1987). The rate of change of an allele frequency at a locus in a non-closed population is a function of the allele frequency, dominance, fitness of the alleles and the population structure (McKenzie 1996). To manage resistance effectively all four factors must be understood. Resistance management techniques include the rotation and mixing of pesticides with different modes of action. These techniques assume that in the absence of selection by a particular pesticide any resistance alleles for that particular pesticide will be disadvantageous and will be lost from the population by either natural selection or dilution by migration (Roush and McKenzie 1987).

There is conflicting data relating to the persistence of resistance alleles in the absence of selection. *Rdl* resistance frequencies in *Drosophila* populations were recorded as high as 1-10% in the apparent absence of cyclodiene selection in an orchard in New York (Aronstein, Ode et al. 1994), although the use of endosulfan may have caused continued low levels of selection. There are several studies that show the loss of the resistant allele in the field when insecticide use is withdrawn. The percentage of dieldrin resistant sheep blowfly, *Lucilia cuprina*, showed a significant decline when treatment with dieldrin was removed (Hughes and McKenzie 1987). In addition, resistant alleles in Turkish acetylcholinesterase resistant *Anopheles sacharovi* decreased in the absence of insecticide selection (Hemingway, Small et al. 1992) and in Italian populations of *Culex pipiens* (Bonning and Hemingway 1991) however in Cuban populations of *C. pipiens* there was no observed drop in resistant acetylcholinesterase allele frequency (Rodriguez, Ortiz et al. 1993).

Resistant alleles are assumed to be pleiotrophic due to the physiological and biochemical changes that are associated with the resistant phenotype (McKenzie 1996). In the case of metabolic resistance, a trade off is assumed to occur between the enhanced ability to detoxify lethal doses of insecticide and a

reduced ability in another fitness component. Large phenotypic modifications are argued to be deleterious within the original context of selection (Coustau, Chevillon et al. 2000). There have been a number of studies that have attempted to determine whether insecticide resistance confers any fitness costs in the absence of insecticide. There has been no general consensus as to whether insecticide resistance does confer a disadvantage in the absence of selection (Coustau, Chevillon et al. 2000). A study by Minkoff and Wilson studied the competitive ability and fitness costs of methoprene resistance in *Drosophila* (Minkoff and Wilson 1992). They found only small differences in life history traits such as the pupal developmental time and pupal mortality between the resistant strains when compared with the wild type. However when the genotypes were competed within population cage experiments the resistant genotype was reduced to 1/10 the original frequency over a period of approximately 150 days. They concluded that small differences in fitness components can have a large effect on the competitive ability of the genotype. In *Anisopteromalus calandrae* no fitness costs associated with malathion resistance were detected; this could be caused by the lack of negative pleiotropic effects associated with the resistant allele (Baker, Perez-Mendoza et al. 1998). Malathion resistance in the red flour beetle, *Tribolium castaneum* has been shown to be correlated with male reproductive success. The absence of a fitness cost may be the result of post-selection of a modifier gene which ameliorate the fitness of resistant individuals or that the resistance gene is non deleterious (Arnaud and Haubruge 2002).

In the mosquito *Culex pipiens* a detailed study of resistance costs has revealed that the costs of resistance are highly variable (Raymond, Berticat et al. 2001). Ace-based OP resistance first appeared in the study area (southern France) in 1972. Resistance genes spread and increased in frequency in the treated area, as well as migrating into the non treated area, where they appeared to be selected against due to their fitness cost. Over a period of 10 years, the replacement of one resistance gene, Ester1, by Ester4, which confers lower levels of OP resistance, indicates the most costly resistance genes are replaced by more recent and less costly ones (Raymond, Berticat et al. 2001). In addition to allelic replacement by a less costly allele, the selection of modifier genes can also ameliorate the cost of insecticide resistance (Coustau, Chevillon et al. 2000). Diazanon resistant Australian sheep blowfly, *Lucilia cuprima,* show increased

levels of asymmetry, a measure of differences between the right and left sides of a bilaterally symmetrical organism (McKenzie and O'Farrell 1993; McKenzie and Yen 1995). These fitness and asymmetry effects are proposed to be the result of developmental instability caused by the introduction of the new resistance allele into the genome. Mutation of a second gene, Modifier (M), resulted in a dominant increase of the fitness of resistant flies. In the absence of diazinon, susceptible and resistant flies had equal fitness and a decrease in the level of asymmetry of resistant flies, returning asymmetry to that of the susceptible flies (McKenzie and O'Farrell 1993).

Among the four molecular mechanisms of insecticide resistance (constitutive overproduction, constitutive underproduction, target modification and inducible change in gene regulation), it is expected that there is no cost of resistance only where resistance results from an inducible change in gene regulation (Coustau, Chevillon et al. 2000). A higher metabolic rate may be necessary for the resistant individuals to maintain the resistance mechanisms unbalancing their gas exchange (Guedes, Oliveira et al. 2006). High body mass and differences in fat body morphology in insecticide-resistant populations suggest the availability of energy reserves for the maintenance of the resistant phenotype without compromising other essential processes. One study of pyrethroid-resistant and susceptible populations of the maize weevil *Sitophilus zeamais* shows that the susceptible population has smaller fat body cells (trophocytes) than resistant populations and the cells from resistant insects have more vacuoles, proteins and carbohydrates than cells from susceptible insects. This suggests that the insecticide-resistant populations have modified the morphology of fat body cells to favour higher stored energy reserves, leading to larger cells (Guedes, Oliveira et al. 2006).

Studies of the potential costs associated with xenobiotic resistance in the absence of the selective agent can suffer from several confounding experimental factors. Laboratory studies look at individual traits such as fecundity, longevity and viability or use population cages to track the frequency of the resistant allele over a number of generations in the absence of selection by insecticide. There are problems with both types of study, primarily the possibility of the artificial environment affecting the fitness of the resistant or susceptible insects and not

providing a true reflection of the fitness of alleles in the field (Houle and Rowe 2003). However the laboratory environment allows the study of fitness under a controlled environment and with laboratory models that give a greater range of genetic techniques. Studies using these techniques have, like the field studies, provided mixed conclusions as to the cost of resistance (Coustau, Chevillon et al. 2000). Fitness costs associated with strains in which resistance has been repeatedly selected for in the laboratory are unlikely to represent fitness costs associated with resistance mechanisms found in the field. The resistant and susceptible strains compared are also often genetically unrelated and any observed costs may therefore be independent of the resistance trait itself. A final confounding factor in the study of the costs of resistance is that when insects are used they are often not checked for the presence of microbial pathogens, such as *Wolbachia*, which can influence the outcome of crosses between infected and uninfected strains.

3.1.2 Prevalence of *Accord* in wild populations

The frequency of the *Accord* element in East African derived populations has been estimated at 32-55% whereas in non-African populations this estimate is 85-100% (Catania, Kauer et al. 2004). DDT use was widely used in the 1940s and 1950s, but use of DDT declined as resistance occurred and in the 1980s use of DDT was widely banned (WHO, 2005). In Africa the highest frequency of the *Accord* was found in West and North African populations at a frequency of 70-90%. This is despite the ban on DDT use in most countries outside of Africa. There are a number of possible reasons why the *Accord* element may be still so prevalent. First, *Cyp6g1* shows broad cross-resistance to organophosphate and carbamate insecticides (Daborn, Yen et al. 2002), which may be selecting for *Cyp6g1* overexpression. Second, *Cyp6g1* is capable of metabolising a number of xenobiotics and endogenous compounds (Daborn, Yen et al. 2002). This may give DDT resistant strains an advantage over the susceptible insects in the field even in the absence of DDT. Since the role of *Cyp6g1* in the wild type insect is not currently known then there is a strong possibility that the role of *Cyp6g1* is in the detoxification of endogenous compounds. Finally, low levels of migration in *Drosophila* and no measurable costs to overexpression of *Cyp6g1* would be

expected to result in no loss of the *Accord* in populations after DDT use is removed.

3.1.3 *Wolbachia*

Wolbachia are gram-negative bacteria that are cytoplasmically inherited rickettsiae found in reproductive tissues (ovaries and testes) of a wide range of arthropods (Werren 1997). *Wolbachia* are transmitted in eggs but are not generally found to be transmitted through sperm (Binnington and Hoffmann 1989). They have so far been found in over 80 insect species, 17 isopods, and a mite (Werren 1997). It has been estimated that 20-75% of all insects may be infected with *Wolbachia* with over 16% of insect species, including each of the major insect orders infected (Werren, Zhang et al. 1995). Much of the success of *Wolbachia* can be attributed to the diverse phenotypes that result from infection. These range from classical mutualism to reproductive parasitism as characterized by the induction of parthenogenesis, feminisation of genetic males and the generation of cytoplasmic incompatibility (CI) in early embryos (Werren 1997).

CI was first detected as a reduction in progeny numbers from crosses between certain strains, and cytoplasmic inheritance was shown in subsequent crosses. *Wolbachia*-induced CI is a reproductive incompatibility between sperm and egg, which typically results in zygotic death in diploid species (Yen and Barr 1971) or male production in haplodiploid species (Breeuwer, Stouthamer et al. 1992). There are two forms of CI, unidirectional incompatibility, which typically occurs when the sperm from a *Wolbachia*-infected male fertilize an uninfected egg, and bidirectional incompatibility, which occurs where the male and female are infected with different strains of *Wolbachia* that are mutually incompatible (Werren 1997). CI is associated with early mitotic defects in the fertilized egg. In the parasitic wasp *Nasonia vitripennis*, it has been shown that the paternal chromosomes form a diffuse chromatin mass in the first mitosis, fail to undergo segregation and typically are lost in later divisions (Breeuwer and Werren 1990). In diploid species such as *D. simulans*, both abnormal first mitosis and later stage disruptions in embryogenesis have been observed. The paternal pronucleus and

associated chromosomes show aberrations in a large percentage of CI-expressing embryos (Lassy and Karr 1996).

Further effects of *Wolbachia* infection include feminisation and the induction of parthenogenesis. Isopod crustaceans have long been known to be affected by cytoplasmically inherited microorganisms that induce feminising (Legrand, Legrand-Hamelin et al. 1987). The best-studied example of feminisation is the woodlouse, *Armadillidium vulgare* (Rigaud, Souty-Grosset et al. 1991). The feminising bacterium in this species acts by suppressing an androgenic gland, thus converting males into reproductively competent females. The induction of *Wolbachia* induced parthenogenesis (the growth and development of an embryo without male fertilization) has been discovered in hymenopterans. It is unknown whether *Wolbachia* induce parthenogenesis outside of the Hymenoptera although the cytogenetic mechanism may bias their distribution to haplodiploid species (Werren 1997). In *Trichogramma spp.*, the first mitotic division, the chromosomes condense as normal in prophase but fail to segregate in metaphase, resulting in the diploidisation of the nucleus (Stouthamer and Kazmer 1994). This mechanism is known as gamete duplication and results in homozygosity at all loci.

There is evidence that *Wolbachia* infection is associated with reduced fitness in *Drosophila*. In *Drosophila simulans, Wolbachia* infection has been shown to be associated with decreased fecundity in females (Hoffmann, Turelli et al. 1990), and decreased fertility in the male (Snook, Cleland et al. 2000). *Wolbachia* has been shown to affect sperm production in male *D. simulans* (Snook, Cleland et al. 2000) and decrease sperm competitive ability in *D. simulans* (Champion de Crespigny and Wedell 2006). High sperm production is considered important in obtaining paternity under sperm competition (Parker 1998). These fitness costs are thought to place selective pressure on hosts to avoid or reduce the effects of *Wolbachia*. There is evidence to suggest that that *Wolbachia* infection is associated with an increased the rate of mating in infected males in both *D. simulans* and *D. melanogaster* (De Crespigny, Pitt et al. 2006). This may serve to increase the spread of *Wolbachia*, or alternatively, may be a behavioural adaptation employed by males to reduce the level of CI. In the study of fitness components in *Drosophila*, it is important to control for potential *Wolbachia*

infection by the antibiotic treatment of strains. Most studies of fitness costs in insects do not take into account the potential effects of *Wolbachia* infection. All strains used in this thesis for life history and reproductive studies were treated with tetracycline to remove any *Wolbachia* infection before life history analysis.

3.1.4 Aims of this chapter

The aims of this chapter are to use *DDT-R* and *DDT-S* strains of *D. melanogaster* to study the fitness effects of *Cyp6g1* over expression in the absence of selection. The frequency of the *Accord* element in East African derived populations has been estimated at 32-55% whereas in non-African populations this estimate is 85-100% (Catania, Kauer et al. 2004). The high frequency of the *Accord* element in non-African populations despite the withdrawal of the use of DDT it may indicate that *Cyp6g1* over expression does not confer any reduction in fitness or that over expression of *Cyp6g1* may confer an advantage even in the absence of DDT. In this chapter two different approaches will be used to study the costs of resistance. First, a life history analysis measuring individual traits associated with fitness and second population cages tracking the frequency of the resistance allele over time. The *Accord* element will be backcrossed into the *DDT-S* strain to allow a direct comparison of the effect of the *Accord* while minimising the differences between the genetic background of the two strains.

3.2 MATERIALS AND METHODS

3.2.1 Generation of backcrossed strain

The standard DDT-susceptible strain of *D. melanogaster* used was Canton-S (referred to as SS). This strain was compared to one in which the *Accord* element had been inserted into a Canton-S background (RR). To generate this strain, Canton-S was crossed with Hikone-R [*Rst(2)DDT*$^{Hikone\text{-}R}$]. The offspring of this cross were then backcrossed with Canton-S for six generations. Selection for those flies with the *Accord* element was performed using 10 µg/vial DDT in standard procedures (Daborn, Boundy et al 2001). Single pair crosses were set up using males and virgin females of the backcrossed line. *Wolbachia* treated and infected strains were obtained from Tim Karr (University of Bath).

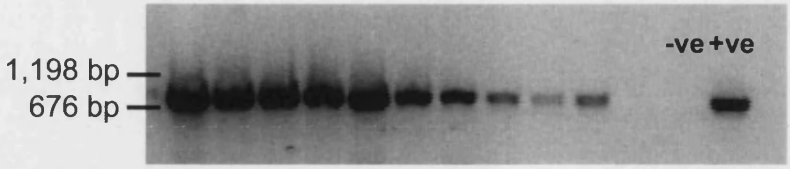
Five generations of backcrossing would replace approximately 98% of the genome of Hikone-R with the Canton-S susceptible genome. The female linked advantage may be due to a closely linked gene and not as a result of *Cyp6g1* over expression. To reduce even further the Hikone-R background with the Canton-S background, the backcrossed strain was backcrossed into Canton-S a further 15 times using the method described above.

3.2.2 *Accord* PCR diagnostic

A PCR diagnostic was performed to identify the presence or absence of the *Accord* element. For DNA extraction, individual flies were homogenized in 50 µl of 10 mM Tris pH 8.2, 25 mM NaCl, 1 mM EDTA, 200µg/ml proteinase K in a 1.5 ml eppendorf tube with a polypropylene homogeniser (Sigma). Homogenates were then incubated at 37°C for 30 min, and 85°C for 10 min.

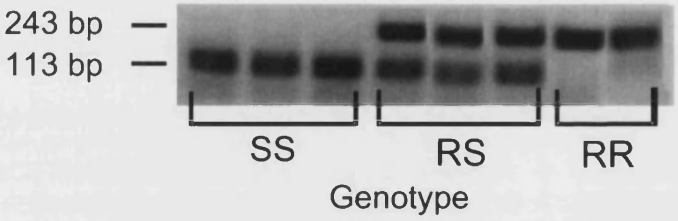
PCR mixtures of 20 µl, containing 1 µl of DNA from single flies, were used for the PCR reaction. The forward primers used were MBWTF – (upstream of *Accord*) and MBFacc 5'-GGG TGC AAC AGA GTT TCA GGT A-3' (Positioned within *Accord*). The reverse primer MBR+6 – 5'-CTT TTT GTG TGC TAT GGT TTA

Figure 3.1 Detection of *Wolbachia* 16s RNA in single flies using PCR.



Presence of Wolbachia *is indicated by a single 700bp band. Positive control is a strain of heavily infected* D. simulans *(Riverside) and a previously treated* Wolbachia*-free strain of* D. melanogaster *(yw) was used as a negative control.*

Figure 3.2 Detection of the presence of *Accord* fragment in single flies using PCR.



The band at 243kb represents the PCR fragment produced when the Accord *element is present and the band at 113kb represents the PCR product generated when the* Accord *element is not present.*

GTT-3' is situated downstream of the *Accord* element. The PCR conditions were as follows: 2 min at 95°C to activate the DNA polymerase, followed by 35 cycles of 95°C for 1 min for denaturation and 55°C for 1 min annealing stage and 72°C for 1 min for the extension phase followed by a final extension step of 72°C for 10 min. 5 µl of the PCR products were run on a 1.5% agarose gel. When the *Accord* element is present a product of 243bp can be observed and when the *Accord* element is absent a product of 113bp can be observed. The offspring of crosses where both the male and female were homozygous for the insertion were used to establish populations of backcrossed flies.

3.2.3 *Wolbachia* diagnostic and treatment

Recent surveys of *Drosophila melanogaster* strains have revealed widespread infection by the intracellular bacterium *Wolbachia* (Werren 1997). As this bacterium can distort sex ratios and reduce progeny in crosses using infected flies we first cured all fly strains used. Both strains were treated for *Wolbachia* by raising one generation on tetracycline fly media (standard fly media with 0.25 g/l tetracycline). Individuals were raised on standard antibiotic media for one generation before any experimental use. The removal of *Wolbachia* from the flies was confirmed by PCR analysis (O'Neill et al 1992). Fly DNA extractions were performed as described above. PCR mixtures of 20 µl, containing 1 µl of DNA from single flies, were used for the PCR reaction. *Wolbachia* 16s RNA was detected using the forward primer Wol16sR-TTGTAGCCTGCTATGGTATAACT and the reverse primer Wol16sF-GAATAGGTATGATTTTCATGT using Reddymix (ABGene) following manufacturers instructions. The PCR conditions were as follows: 72°C for 10 min followed by 30 cycles of 95°C for 1 min for denaturation and 52°C for 1 min annealing stage and 72°C for 1 min for the extension phase. 5 µl of the PCR products were run on a 1.5% agarose gel. When *Wolbachia* is present a band of 700 bp can be observed (figure 3.1). A strain of heavily infected *D. simulans* (Riverside) was used as a positive control and a previously treated *Wolbachia*-free strain of *D. melanogaster* (*yw*) was used as a negative control.

3.2.4 Life history analysis

The fitness of Canton-S (*SS*) and the backcrossed line (*RR*) was analysed by looking at individual fitness traits. These were the mass, fecundity, egg, larval and pupal viabilities, lifespan and developmental rate.

3.2.4.1 Fly mass

To determine if there is a difference in the body size of the flies of the *RR, RS, SR* and *SS* genotypes, which could have an effect on the performance of the genotypes in the fitness studies the dry mass of the flies was calculated. Five sets of male and female flies of each genotype were collected. Each set of flies consisted of 50 individuals. The flies were frozen briefly at -80 °C to kill the flies. The flies were wrapped in foil and dried overnight in a hybridisation oven at 50°C. A microbalance was used to weigh the sets of flies. Data were recorded in Microsoft Excel and statistical analyses performed in MINITAB 12.0. Data were analysed using analysis of variance (ANOVA) and Tukey's post hoc test.

3.2.4.2 Fecundity assay

The total lifetime fecundity of *SS* and *RR* genotypes was compared. Flies were reared at low density on standard treacle media. Virgin flies were collected and kept at a density of approximately 20 flies per vial until used. Single pairs of three-day-old non-mated flies were lightly anaesthetised on CO_2 then placed in 10 mm shallow petri dishes containing 30 ml egg laying agar spread (appendix A2) with a thin layer of 10% (w/v) live yeast solution. A fresh yeast solution was used each day. All plates were seeded with 500 µl yeast from a single batch and then left until the water evaporated to prevent flies sticking and drowning in the liquid. Plates were kept at room temperature and under constant light conditions (the conditions the strains are reared at).

After 24 hours the flies were anaesthetised with CO_2 and transferred to a new plate. The number of eggs on each agar plate was recorded. The fecundity of

female *RR* was measured when mated to male *SS* and male *RR*. The fecundity of female *SS* was measured when mated to male *RR* and male *SS*. Twenty repeats were performed for each cross. Flies that died within the first three days were replaced with an unmated fly of the same age. After 20 days the flies were turned over every 72 hours until one of the flies in the cross died.

The fecundity of RR and SS was compared with the fecundity of the offspring of the crosses RR x SS and SS x RR to determine if the higher fecundity is a dominant trait. Pairs of flies were placed in customised egg collecting manifolds. Each female was placed in a separate chamber of the manifold with a single 3-5 day old male Oregon-R and the pairs were observed to ensure mating. Any pairs not mated within 20 min were replaced and the new pairs observed for mating. Manifolds were incubated at 25°C (+/- 1°C) under constant light. The agar laying surface was replaced daily and the number of eggs laid with the 24 h period were counted for five days. This was repeated at 20 °C to see if the temperature has any effect on the fecundity of the females. Data were recorded in Microsoft Excel and statistical analyses performed in MINITAB 12.0. Data were analysed using analysis of variance (ANOVA) and Tukey's post hoc test.

3.2.4.3 Crowding effects on fecundity

To determine if the observed difference in fecundity is still observed under crowded conditions virgin female *SS* and *RR* were collected and placed in vials with males of each strain. The pairs were watched for up to 20 min until mated and 20 mated pairs introduced into egg collecting chamber consisting of a 50 mm shallow petri dish with a layer fruit juice agar spread with 1% live yeast solution. One end of a 50 ml polypropylene vial was attached to the lid of the petri dish and hole cut in lid to allow the flies to fly freely and lay on the agar. The other end of the vial was removed and gauze was glued over the end allowing a free flow of air into the chamber. The base of the chamber was replaced with one containing a fresh agar and yeast layer every 24 h. The number of eggs laid on each plate was counted daily for 5 days. The chambers were incubated under constant light at 25°C. Data were recorded in Microsoft Excel and statistical

analyses performed in MINITAB 12.0. Data were analysed using analysis of variance (ANOVA) and Tukey's post hoc test.

3.2.4.4 Viability assay

Approximately 40 males and females of each cross were allowed to mate and lay on egg laying agar. The crosses were as follows *RR x RR, RR x SS, SS x RR* and *SS x SS*. Eggs were washed from the plates and approximately 100 of each cross were added to five half-pint bottles with 50 ml standard fly food. Each bottle had an added tissue to absorb excess moisture. The mean weight of the males and females of each strain was determined by weighing 20 females from each bottle.

Manifolds were prepared with 1 ml fruit juice agar spread with 10 µl 10% (w/v) live yeast solution. Flies were added and observed for mating. Manifolds were kept at room temperature under constant light. The manifolds were turned over every 24 hours. The number of eggs was counted after 24 h. The egg viability was recorded as the number of eggs hatched 48 h after the flies were turned over. 20 larvae were transferred from each cross into a vial containing standard fly food media. The viability of these larvae was recorded as the number forming pupae. Pupal viability was recorded as the number of flies emerging from pupae within 20 days of the first fly emerging. The post-emergence weight of these flies was recorded. Data were recorded in Microsoft Excel and statistical analyses performed in MINITAB 12.0. Data presented as percentages were transformed using the arc-sine square root transformation and analysed using analysis of variance (ANOVA) and Tukey's post hoc test.

3.2.4.5 Developmental rate

There have been a small number of studies indicating that resistance may affect developmental rate (Minkoff and Wilson 1992; Fragoso, C. et al. 2005). To investigate this using the backcrossed strain four crosses of the flies were established (*RRxRR, RRxSS, SSxRR* and *SSxSS*) and were allowed to lay on

egg laying agar plates spread with 10% (w/v) yeast in water for 2 h to encourage female to lay all embryos. After the "egg expulsion" the flies were moved to new egg laying plates and left to lay for 30 min. These embryos were washed from the plates with Egg Washing Buffer (appendix A4) and transferred with a fine paintbrush to food vials. 20 embryos were transferred per vial and these vials stored at either 20ºC or 25ºC for the length of the experiment. The embryos were checked after 48 h and then monitored every 12 h for pupae. Once larvae had begun pupation the time was recorded and the outside of the vial marked to indicate the order of pupation. The vials were monitored every 12 h until 10 days after the first fly emerged. Any pupae which were remaining were considered to be non-viable after this period of time. Data were recorded in Microsoft Excel and statistical analyses performed in MINITAB 12.0. Data were analysed using analysis of variance (ANOVA) and Tukey's post hoc test.

3.2.4.6 Lifespan

Flies were allowed to lay in standard food vials for 2 h. Four crosses were established, *RRxRR, RRxSS, SSxRR* and *SSxSS*. The vials were stored at 25ºC until pupae turned dark. Virgin flies were collected over an 8 h period and these flies were stored in male only and female only vials with 20 flies per vial. The flies were moved to a new food vial every 8-10 days and the number dead counted daily. Data were recorded in Microsoft Excel and statistical analyses performed in MINITAB 12.0. Data were analysed using analysis of variance (ANOVA) and Tukey's post hoc test.

3.2.4.7 Increased number of backcrosses

Five generations of backcrossing would replace approximately 98% of the genome of Hikone-R with the Canton-S susceptible genome. The female linked advantage may be due to a closely linked gene and not as a result of *Cyp6g1* overexpression. To reduce even further the Hikone-R background of the backcrossed strain, the original backcrossed strain was backcrossed for an additional 15 generations using the method described in 3.2.1.

3.2.5 Population Cage Studies

Population cages were established with males and virgin females from the backcrossed line (*RR*) and the DDT susceptible strain Canton-S (*SS*). Cages were established in half pint glass bottles containing 60 ml of treacle fly food. A tissue was added to absorb excess moisture.

Three replicate cages were established with 50 male *RR* and 50 female *SS* and three replicates were established with female *RR* and male *SS* (figure 3.3). The flies were allowed to mate and lay eggs for 72 h after which time the cages were emptied to limit larval density. The cages were emptied seven days post emergence of the first flies from the pupal cases. The emergent flies from within the day collection period were used to establish the next cage. After the following 72 h laying period the cages are emptied and the adults are genotyped using the *Accord* PCR diagnostic. For DNA extraction, single flies were homogenized in homogenisation buffer (see 3.2.2) in a 96 well plate (Nunc) with a customised 96 well homogeniser. Homogenates were incubated at 37°C for 30 min, and 85°C for 10 min. The PCR diagnostic was performed using the primers and reaction temperatures described above and using Reddymix PCR mastermix (ABGene). 1 µl DNA was used in a 20 µl reaction. The PCR products were run on a 1.5% agarose gel (figure 3.4) and the genotypes of the flies were determined. In order to show results from the initial cages were not affected by the frequency of emptying the cage, further population cages using the methods described above were performed with rates of empty of 3 and 5 days post emergence.

Figure 3.3 Population cage protocol

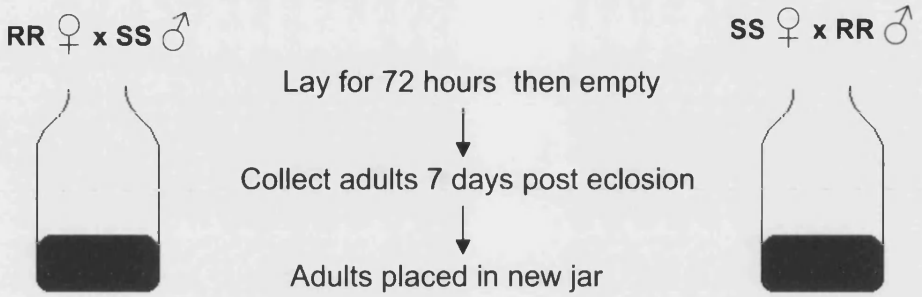
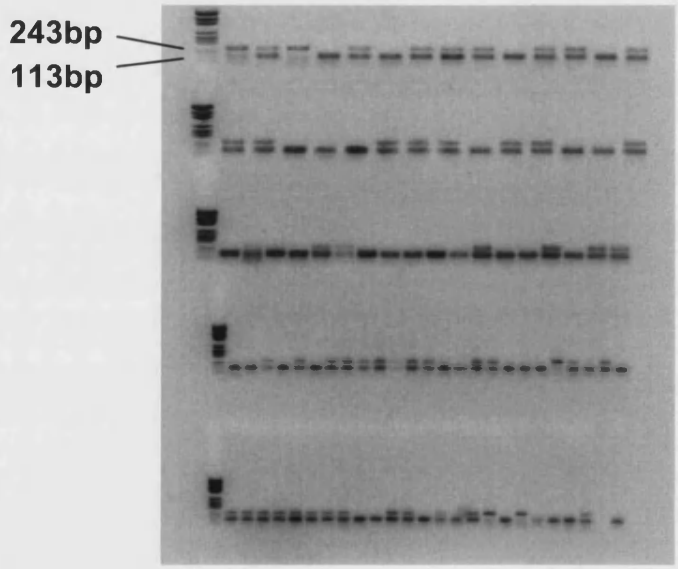


Figure 3.4 Example of a gel showing the products of PCR amplification of single flies from a single generation of the population cages.



The band at 243kb represents the PCR fragment produced when the Accord *element is present and the band at 113kb represents the PCR product generated when the* Accord *element is not present.*

3.3 RESULTS AND DISCUSSION

3.3.1 Life history analysis

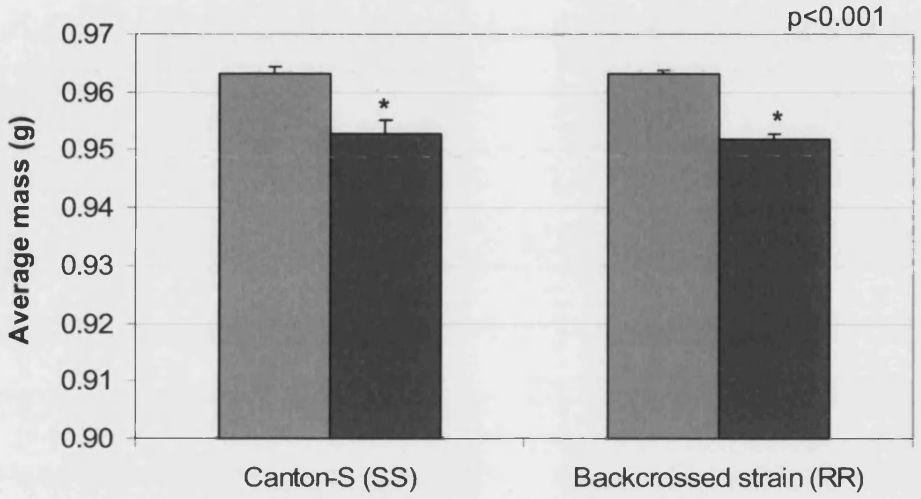
3.3.1.1 *Wolbachia*

All the *Drosophila* strains used in this study were successfully cured of *Wolbachia* and it is therefore possible exclude *Wolbachia* infection as a basis for fluctuations in resistance frequencies in the population cages and variation in fitness traits in the life history study. After treatment for *Wolbachia*, the strains were raised on non-antibiotic media for two generations to exclude the possibility of the antibiotic treatment affecting the life history analysis. Many other studies of the fitness costs of insecticide resistance do not control for possible *Wolbachia* infection in one or more of the experimental strains and therefore the results may be affected by the fitness costs of *Wolbachia* infection.

3.3.1.2 Adult mass

The mass of flies has been shown to correlate with fitness and competitive ability (Ewing 1961; McCabe and Partridge 1997). Increased female size is associated with longer lifespan and increased offspring production (McCabe and Partridge 1997). To ensure that the life history analysis was not affected by the mass of the flies, both strains were raised on identical diet, at low densities. There was no significant difference between the dry mass of female *SS* and female *RR* or between male *SS* and male *RR* ($F= 96.50$, $p< 0.001$) raised under uncrowded conditions (figure 3.5). As would be expected, female *SS* and female *RR* have a significantly higher body mass than both male *RR* and male *SS*. Other traits may be used to assess body size; traits that are often measured are the length of the wing or hind leg. Although just adult body mass was used in this study, it is seems that any fitness differences due to differences in the resources available to the flies are minimal.

Figure 3.5 Dry mass of Canton-S (*SS*) and Backcross (*RR*) flies



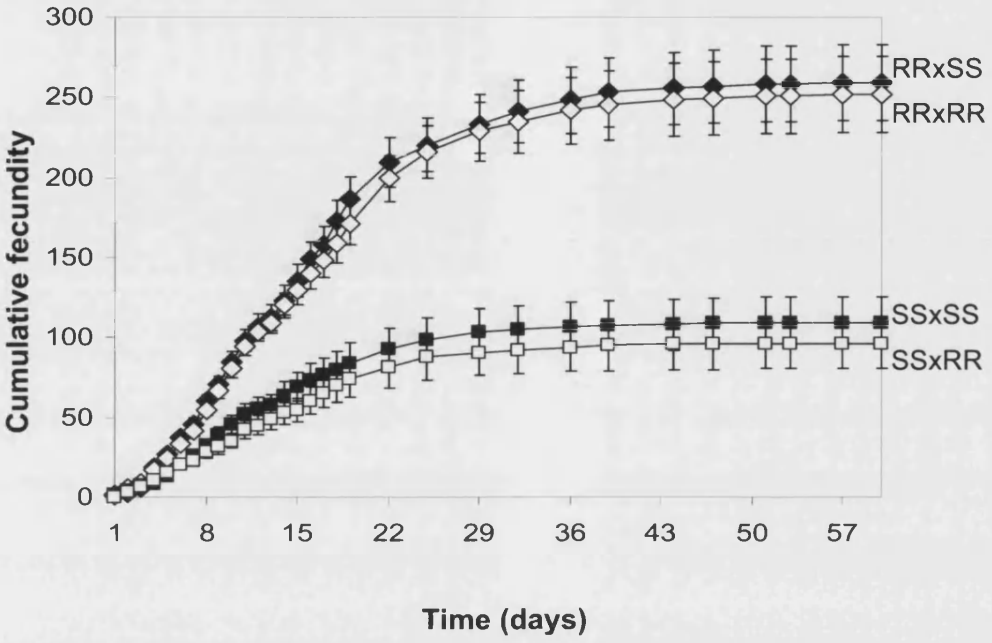
*The dry mass of 20 flies was calculated, females are indicated in light grey and males in dark grey. * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons) Error bars are plotted indicating standard error.*

3.3.1.3 Fecundity

The fecundity of female RR is significantly higher ($F= 19.15$, $p< 0.001$) than the fecundity of female SS (figure 3.6). The higher fecundity of RR is not affected by the genotype of the male that the female is mated to. The cumulative result of this is that the total lifetime fecundity of the female flies homozygous for the *Accord* element is on average double the total lifetime fecundity of flies with no copies of the *Accord* element (figure 3.6). To determine whether the higher fecundity of females carrying the *Accord* is dominant, females of genotypes *RR, RS, SR* and *SS* were mated to males of the strain Oregon-R. The unrelated strain Oregon R was chosen as the male strain to prevent and effects of relatedness affecting the differences in the fecundity of the females. The results show that over the five-day egg laying period the fecundity of the female with the *Accord* element is on average higher than the fecundity of the female without the *Accord* element ($F= 18.17$ $p< 0.001$), indicating that the higher fecundity is dominant (figure 3.7). This is interesting as the resistance phenotype is also dominant. The over expression of *Cyp6g1* appears to result in higher fecundity in the female.

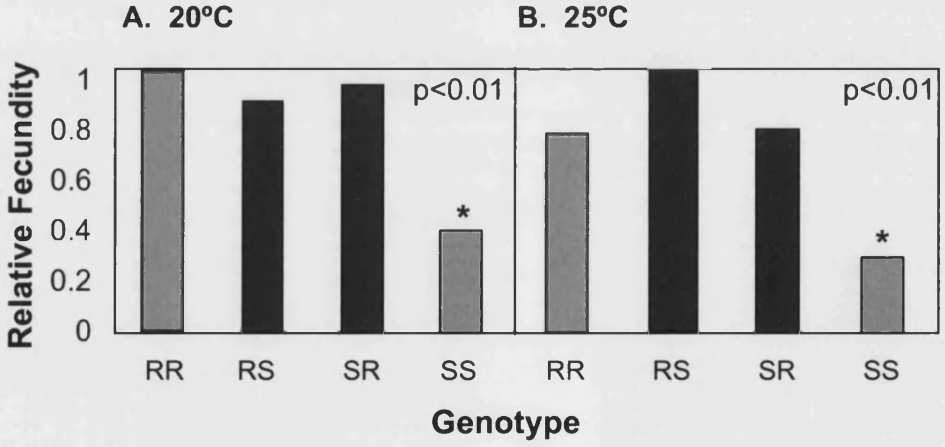
The striking differences in the fecundity of the strains are surprising since no study of insecticide resistance has previously found such marked differences between resistant and susceptible strains. Many other studies do not however control fully for background effects. It is not possible to exclude the possibility that the differences in fecundity are due to another closely linked gene. Backcrossing for six generations would result in approximately 98% similar background between *RR* and *SS*. To reduce this further and to reduce the chances of closely linked genes being responsible for differences in fecundity further backcrosses were performed. These results are described in 3.3.1.8. The choice of Canton-S as a susceptible background may not have been the most suitable as Canton-S has been kept in the laboratory for many years and is likely to have undergone many population bottlenecks and high levels of inbreeding.

Figure 3.6 Cumulative fecundity over female lifespan of Canton-S (*SS*) and Backcross (*RR*) when mated to males of *RR* and *SS* genotypes



The cumulative fecundity of each cross is plotted over the lifetime of the female fly. Error bars are plotted indicating standard error

Figure 3.7 Fecundity of different *DDT-R* genotypes over five day egg laying period at two different temperatures



*The fecundity of each genotype is plotted relative to the most fit which is given a value of 1.0, a * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons).*

The backcrossing of the *Accord* was attempted using strains recently collected (within one year) from wild populations. The strains were kindly donated from collections made by Todd Schlenke in California. After identifying a strain that lacked the *Accord* insertion, an attempt was made to backcross the *Accord* into the genome of this strain. The strains did not survive well on artificial diet however and were lost during the backcrossing process. With more time and a range of diets it would be possible to backcross the *Accord* into a wild background. Furthermore the comparison of life history traits of non-*Accord*, field derived strains with *Accord* field derived strains may be useful in supporting the results obtained with the backcrossed line, although this method does not account for background genetic effects.

Table 3.1. Fecundity summary statistics

Cross	Mean eggs laid/day (+/- SE)	Mean total eggs laid (+/- SE)
RR female x RR male	10.1 (+/- 1.5)	259.5 (+/- 23.7)
RR female x SS male	9.3 (+/- 1.5)	251.9 (+/- 23.8)
SS female x RR male	4.7 (+/- 1.6)	109.2 (+/- 16.3)
SS female x SS male	4.4 (+/- 1.1)	96.1 (+/- 15.7)

3.3.1.4 Viability of lifestages

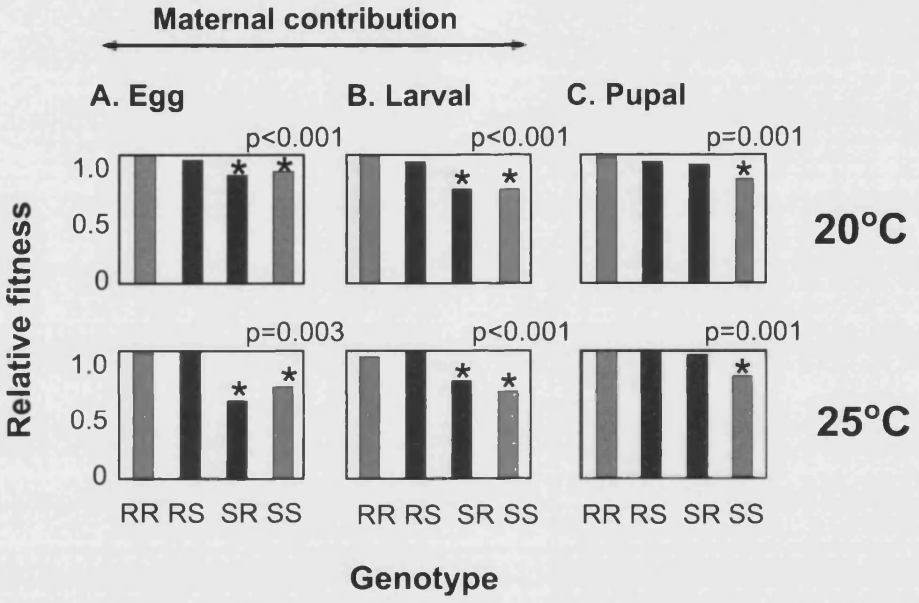
The viability of the eggs, larvae and pupae were measured under uncrowded conditions at 25°C. The viability of the eggs laid by *RR* females over the lifetime of the fly was significantly higher ($F= 7.51$, $p< 0.001$) than the viability of eggs laid by *SS* females regardless of the genotype of the male that the female is mated to (figure 3.8 A). The number of eggs hatching was slightly lower than might be expected and may indicate suppression under prolonged laboratory conditions. The survival of larvae of *RR* females was significantly higher ($F= 7.70$, $p<0.001$) than the survival of larvae laid by *SS* females, regardless of the genotype of the male parent (figure 3.8 B). The viability of the pupae of *SS* females was significantly lower ($F= 5.57$, $p= 0.001$) than the viability of the pupae of *SS* female that have been mated to *RR* males or *RR* females mated to either *SS* or *RR* (figure 3.8 C). There was no significant difference between the viability of pupae with one or two copies of the *Accord* element. The viability of the life stages was

then measured at 20°C to determine whether the fitness differences were temperature dependant. The viability of the eggs laid by *RR* females over the lifetime of the fly was significantly higher (F= 5.72, p= 0.003) than the viability of eggs laid by *SS* females regardless of the male genotype (figure 3.8 A). The survival of larvae of *RR* females was significantly higher (F= 10.28, p<0.001) than the survival of larvae laid by *SS* females, regardless of the male genotype (figure 3.8 B). The viability of the pupae of *SS* females was significantly lower (F= 6.45, p= 0.001) than the viability of the pupae of *SS* female that have been mated to *RR* males or *RR* females mated to either *SS* or *RR* (figure 3.8 C).

There is indication of a maternal contribution towards the embryo and larvae from the resistant female parent. This maternal contribution results in the higher survival of the embryos and larvae of *RR* female flies. The higher survival is regardless of the male parental genotype. The embryos of resistant male flies that have a susceptible female parent show lower survival, despite the presence of the *Accord* in these embryos and larvae. The pupae however show higher survival when either parent is resistant. The presence of the *Accord* in the pupae is enough to confer higher survival in the RR, RS and SR pupae compared with the SS pupae. These results indicate that a maternal contribution to the embryo is important until the pupal stage and that the overexpression of *Cyp6g1* in the pupae confers higher a higher pupal viability.

The transmission of mRNA and protein from the female to the embryo has been well documented in *Drosophila* for a number of genes (Dworkin and Dworkin-Rastl 1990). Genes expressed during oogenesis and stored in the embryo control early development. These genes may be expressed only maternally, maternally and embryonically, or only embryonically. It is estimated that there are 500-1,000 genes required for oogenesis in Drosophila, but fewer than 100 are exclusively required for the formation of the egg, as opposed to general cellular survival (Perrimon, Mohler et al. 1986). Information may be of two types. First, genes specifying general metabolic processes required for normal development and second, that required for specific early developmental processes although the types of information are not necessarily mutually exclusive (Dworkin and Dworkin-Rastl 1990). The genetic control of segmentation in the embryo involves a cascade of gene regulation beginning with

Figure 3.8 Life history analysis of the different *DDT-R* genotypes at two different temperatures.



*The fitness of each genotype is plotted relative to the most fit which is given a value of 1.0, a * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons) and individual P values are given above each histogram.*

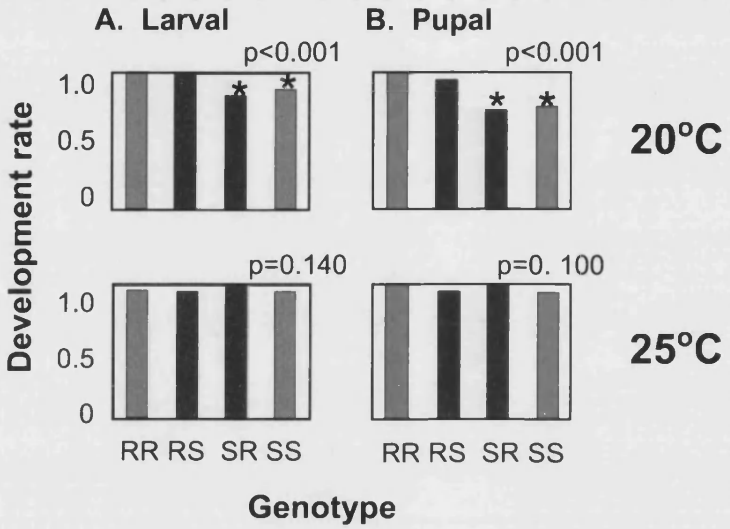
the diffusion of spatially localized maternal RNA (e.g. bicoid, nanos, caudal), from the anterior and posterior poles of the embryo. These control the spatial patterns of transcription of the gap genes regulated by zygotic expression (e.g. hunchback. Krüppel, knirps). The possible transmission of *Cyp6g1* by resistant females to their offspring resulting in increased CYP6G1 may be conferring the increased viability of the embryos. Since CYP6G1 has a broad range of substrates and the wild type function is unknown, a role in oogenesis is possible. This will be addressed further in Chapter 4.

The viability studies indicate a fitness advantage in the strains over expressing *Cyp6g1* however the percentage egg hatch is slightly lower than may be expected for wild type *Drosophila* lines. This is likely to be a result of the laboratory conditions. It is possible that differential rates of fertilisation between the genotypes could be masking the viability of the eggs laid by the two strains i.e. the lower recorded viability of the eggs may be due to a lower rate of fertilisation. To overcome this potential problem, fertilisation assays could be performed allowing the percentage of fertilised eggs laid by *SS* and *RR* to be factored into the analyses.

3.3.1.5 Developmental rate

The developmental rate of the larvae and pupae were measured at two different temperatures, 20 and 25°C. The developmental rate of the larvae of *RR* and *RS* females was significantly faster ($F= 27.66$, $p<0.001$) than the developmental rate of larvae laid by *SS* and *SR* females, (figure 3.9 A) at 20°C. There is no difference in the developmental rate of larvae from any of the four genotypes (figure 3.9 A) at 25°C ($F= 1.90$ $p= 0.140$). The developmental rate of the pupae of *RR* and *RS* females was significantly faster ($F= 6.45$, $p< 0.001$) than the developmental rate of pupae laid by *SS* and *SR* females, (figure 3.9 B) at 20°C. There is no difference in the developmental rate of the pupae of all four genotypes (figure 3.9 B) at 25°C ($F= 11.46$, $p= 0.100$).

Figure 3.9 Developmental rates of the different *DDT-R* genotypes at two different temperatures.



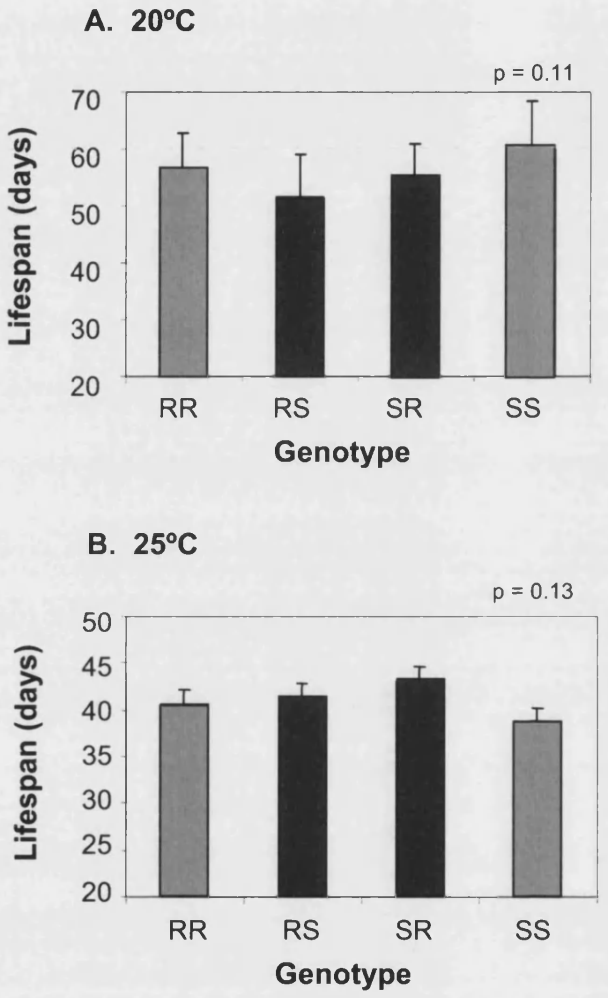
*The developmental rate of each genotype is plotted relative to the most fit which is given a value of 1.0, a * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons) and individual P values are given above each histogram.*

These results are in contrast to the viability studies, which indicate a maternal contribution to the embryo that confers an advantage in the embryo and larvae but is not evident in the pupae. In the case of the developmental rate, there is an increased rate of development at 20°C of the genotypes where the female parent is resistant at both the larval and pupal stages indicating the putative maternally derived factor increases the developmental rate right through until the adult stage. This difference is not evident at 25°C. The lack of any differences at 25°C may be due to the faster development at 25°C masking any difference between the strains. Alternatively as these populations are routinely kept at 25°C, by reducing the temperature to 20°C, an additional stress may be placed on the flies that more detrimental to the flies not gaining the maternal advantage.

3.3.1.6 Lifespan

The lifespan of the virgin flies of the genotypes *RR, RS, SR* and *SS* was measured at two different temperatures, 20 and 25°C. There was no significant difference in the lifespan of the genotypes at either 20°C ($F= 16.80$ $p= 0.11$) or 25°C ($F=22.37$ $p= 0.13$). These results indicate that the over expression of *Cyp6g1* does not affect the longevity of the resistant flies which is interesting to note since it may be expected that the faster developmental rate of the *RR* and *RS* at 20°C would result in a shorter lifespan. The difference in the mean time to adult is just over 24 h so given the lifespan study calculated the mortality of the flies daily, the difference over the 60-70 day lifespan is not significant. Several studies support the idea of a cost of reproduction and courtship in terms of decreased adult lifespan in *D. melanogaster* (Cordts and Partridge 1996; Sgro and Partridge 1999). The trade off between lifespan and fecundity is partly due to both traits sharing resources, thought to be lipids (Zwaan, Bulsma et al. 1995; Chippindale, Leroi et al. 1997) and has been explained by a model of resource allocation with the organism having to allocate limiting lipid reserves to either reproduction or somatic maintenance (van Noordwijk and de Jong 1986). In the case of *DDT-R* there does not appear to be a cost in terms of decreased lifespan with the higher fecundity, however this study used virgin male and female flies.

Figure 3.10 Lifespan of the different *DDT-R* genotypes at two different temperatures.



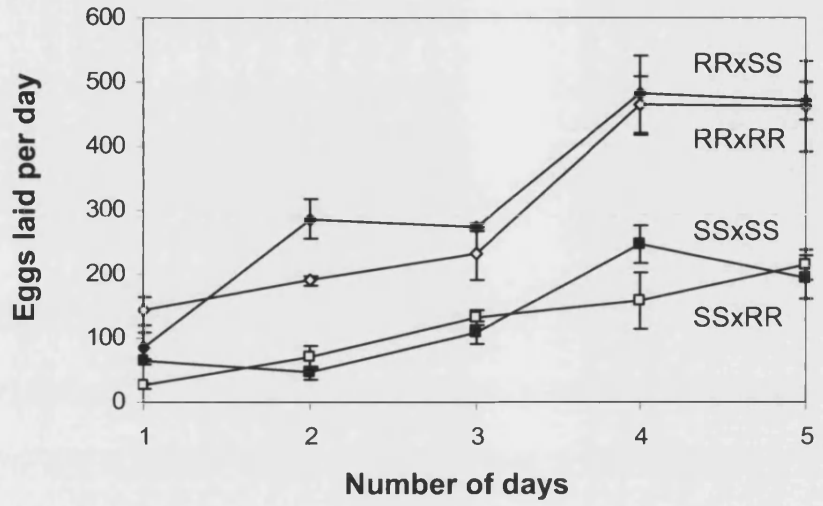
The lifespan of each genotype is plotted with errors bars indicating standard error. The differences between the genotypes are not significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons).

3.3.1.7 Effect of crowding on fecundity

Due to their small size and large surface-to-volume ratio, terrestrial insects are especially vulnerable to such environmental stress and must employ strategies of resistance to promote short-term survival (Albers and Bradley 2006), however, these strategies cannot always be carried out without expense to another fitness variable. To determine whether the fecundity of the *Accord* element flies is higher than the non-*Accord* carrying flies under conditions more closely related to those in wild conditions SS and RR flies were introduced into crowded chambers and the eggs laid under these conditions counted. As seen before, the fecundity of RR females was significantly higher than the fecundity of SS females ($F= 10.69$, $p< 0.001$). The number of eggs laid per fly per day was on average 2.3 times higher in RR regardless of the genotype of the male flies to which the females were mated (figure 3.11) indicating under crowded conditions, with high competition for mates, the fecundity is still higher.

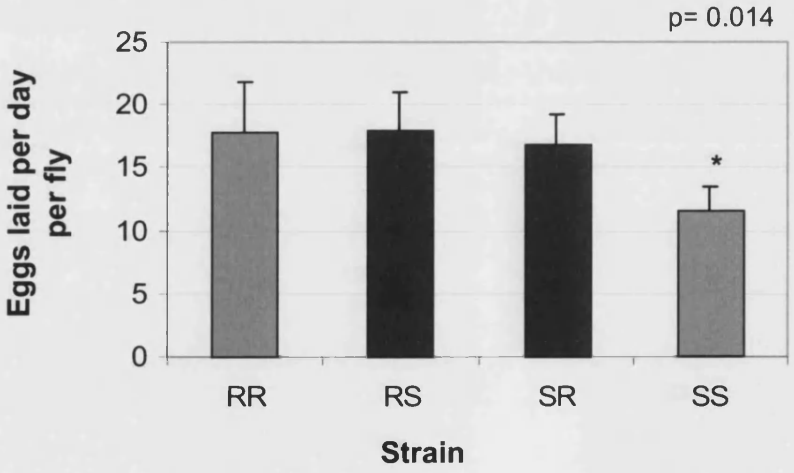
Crowding and limited resources have a number of effects on life history traits. It is has been established that in insects larval crowding reduces the body size of adults due to high larval competition (Baldal, van der Linde et al. 2005). A reduction in adult size is often equated with a decrease in adult fitness and there is strong evidence in insects for a direct and positive association between size and reproductive fitness when measured as female fecundity (Honek, 2003). In *Drosophila*, it has been shown that larval rearing density can also influence adult longevity, heat stress resistance, and Hsp70 production tend to be relatively higher when larvae are reared at an intermediate versus low density (Sorensen and Loeschcke 2001). The accumulation of waste products e.g. urea and ammonia increase as larval density increases, and these presence of these products can interfere with metabolic functions in adults independent of any effects on size (Borash and Ho 2001). *D. melanogaster* larvae with higher levels of parasitoid resistance towards the larval parasite *Asobara tabida* display reduced competitive ability when compared with larvae which have not been selected for parasitoid resistance (Kraaijeveld and Godfray 1997; Kraaijeveld, Ferrari et al. 2002). The effect of *DDT-R* on the competitive ability of the larvae should be quantified, as this is an important fitness trait in the survival of the larvae.

Figure 3.11 Fecundity of different *DDT-R* genotypes over five day egg laying period under crowded conditions



The cumulative fecundity of each cross is plotted over the five day laying period. Error bars are plotted indicating standard error

Figure 3.12 Fecundity of different *DDT-R* genotypes with 20 generations backcrossing over five day egg laying period



*The eggs laid per day for each genotype is plotted with errors bars indicating standard error. A * indicates a significant difference between the genotypes at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons).*

3.3.1.8 20 generation backcross

The fecundity results in 3.3.1.3 compared the fecundity of females with a susceptible background, differing in the presence of the *Accord* element. The potential problem with the backcross was that with 6 generations of backcrossing is that closely linked genes are not eliminated in the backcross and that the differences in fecundity between the resistant (RR, RS, SR) and susceptible SS genotypes is not due to the overexpression of *Cyp6g1.* To address this, the backcrossed strain was subjected to further 14 generations of backcrossing in order to replace the resistant background with the susceptible background. The results show that the fecundity is still higher in the RR, RS and SR genotypes compared with the SS ($F= 4.03$ $p= 0.014$). The difference in the fecundity is not as great as was seen for the six-generation backcross.

3.3.1.9 Conclusions from life history analysis

In summary the results from the life history studies reveal that flies with the *Accord* element show higher fecundity than flies with no *Accord* insertion, this difference is still evident even after 20 generations of backcrossing the insertion into a susceptible background indicating that the presence of the *Accord* is conferring increased fecundity. The strain used as a susceptible strain, Canton-S, has been kept under laboratory conditions for many years and is likely to show inbreeding depression. By backcrossing for 20 generations it is hoped that as much as possible of the background genome of Hikone-R is replaced with the genetic background of Canton-S. It is possible that the selection process inadvertently selected for increased fitness as well as DDT resistance. Repeating the backcrossing with strains recently isolated from the wild would allow the comparison of strains that have not undergone many generations of selection for survival in the laboratory.

The viability studies reveal a maternal contribution appears to increase survival of the embryos and larval until pupal stage at which point the possession of the *Accord* element, whether inherited from the male or female parent seems to confer an increased viability. The developmental rate of the genotypes does not

show significant differences at 25°C but at 20°C the rate of development is greater when the *Accord* element is inherited from the female parent, again indicating a maternal contribution to development. The optimum temperature for *Drosophila* growth is 28°C, at 20°C the maternal contribution or higher fitness of the developing fly may be enabling accelerated growth. Alternatively, since the rate of development is faster at 25°C, the differences between the genotypes are less pronounced. Maternal contributions to the embryo are common in *Drosophila.* Development in early embryos is influence by maternal mRNA and protein passed from the female parent to the embryo (Dworkin and Dworkin-Rastl 1990). To determine whether the female associated increased viability is due to *Cyp6g1* much further work is required. This will be addressed in more detail in Chapter 4.

Life history studies provide interesting results and are an indicator of fitness differences between genotypes. There are however problems with life history studies. In this study the genotype of the individual embryos larvae pupae were not tested. Ideally the genotype of each embryo, larva and pupa would be determined using the *Accord* PCR diagnostic to ensure the results are a true reflection of the fitness of the genotypes. Life history studies can only include a finite number of traits that can have an effect on the fitness of the flies. This study did not look at sexually selected traits; however a large range of naturally selected traits were included. Diazanon resistant Australian sheep blowfly, *Lucilia cupma,* have increased levels of asymmetry, thought to be caused by the introduction of the new resistance allele into a genome (McKenzie and O'Farrell 1993; McKenzie and Yen 1995). This study shows that fluctuating asymmetry in wing length is an important fitness trait in postcopulatory sexual selection in *Drosophila.* Fertilization success is negatively related to the fluctuating asymmetry of wing length, suggesting either female preference for more symmetrical males or a relationship between male asymmetry and intrasexual selection, which can be reflected in mating performance (Otronen 1998; Polak and Starmer 2005). Laboratory conditions are not ideal since they do not recreate the wild environment and do not necessarily give an accurate representation of how these traits would affect survival in wild populations. Under natural conditions there are limited resources and competition is high. Resistance to parasitoid infection in *Drosophila* is correlated with decreased

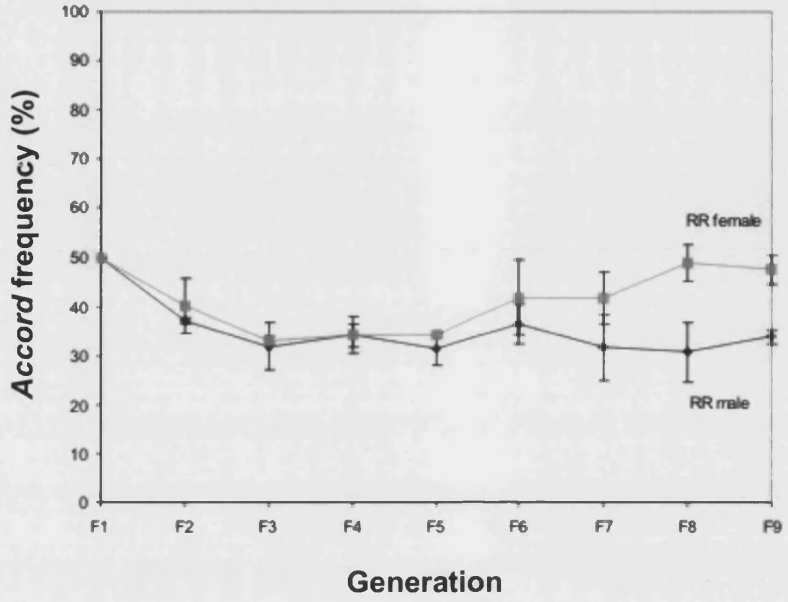
larval competition (Kraaijeveld and Godfray 1997; Kraaijeveld, Ferrari et al. 2002). Further study should test the life history traits under conditions with high competition for resources. An alternative to studying individual traits is to use population cage studies. The population cage approach was also used to look at competition between the genotypes under crowded conditions and in addition to give an indication of the overall effect of any fitness differences in the genotypes.

3.3.2 Population cages

The PCR based diagnostic for DDT-R gave unambiguous and repeatable genotypes for individual flies sampled from the population cages (figure 3.4). This molecular diagnostic allows the calculation of the frequency of the resistance allele with precision and the standard errors of the mean for each set of cages are correspondingly small (figure 3.13). If the overall frequency of the *Accord* element in the two sets of cages over time is plotted, we see an initial decline in the frequency of resistance over the ten generations with little difference between the female x male and male x female cages (figure 3.13). The drop in the frequency of the *Accord* stabilises after the third generation at which point the results from the two sets of cages diverge. In the cages established with resistant females the frequency of the *Accord* begins to increase and after eight generations is up to the 50% starting frequency. In the cages established with resistant males, the *Accord* frequency fluctuates around 35%. This apparent divergence is surprising, as the differences between the cages would be expected to be most evident in the first few generations.

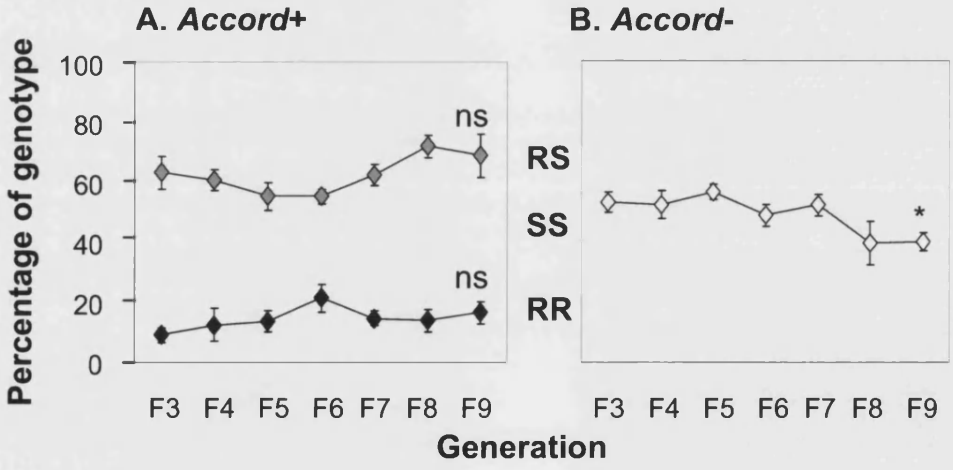
When the frequencies of the resistant (figure 3.14 A) and susceptible (figure 3.14 B) genotypes are plotted, the resistant homozygotes (*RR*) are always the least abundant and heterozygotes (*RS*) are typically most abundant. To understand this difference in behaviour between the *RR* and *RS* genotypes, the expected versus the observed frequency of each genotype were calculated using predictions based on Hardy-Weinberg equilibria (figure 3.15). From this figure it is clear that the RS genotypes are performing as expected but that the RR flies are less fit than expected (X^2= 141, d.f.= 3). This indicates that RR flies carry a fitness cost that is not expressed in heterozygous condition.

Figure 3.13 Frequency of resistance allele over ten generations in the two sets of population cages.



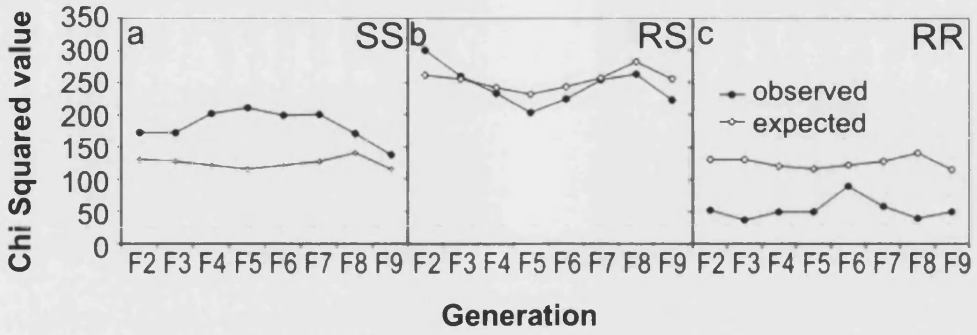
The first set of three cages was started with RR females and SS males (RR x SS) and the second set with SS females and RR males (SS x RR). Cages were emptied five days post eclosion

Figure 3.14 Change in the frequencies of the three *DDT-R* genotypes (*SS, RS* and *RR*) over time in the population cage experiment.



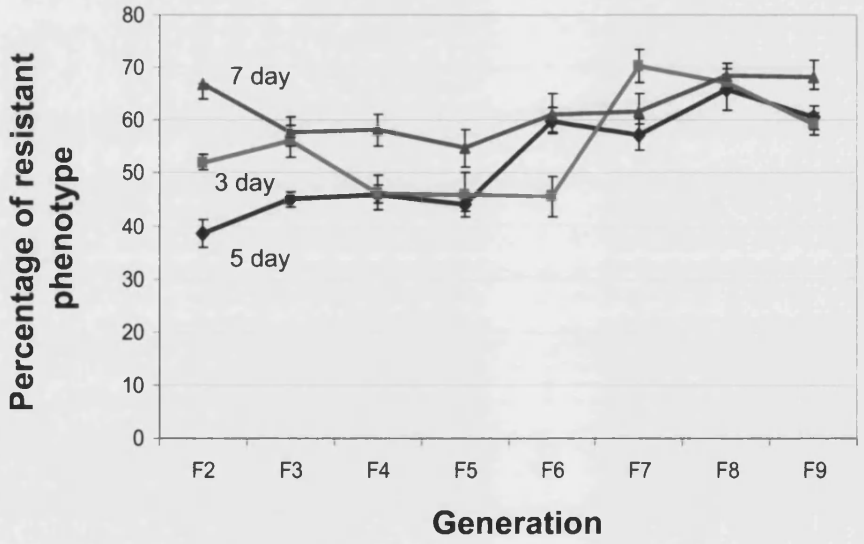
*Starting population frequencies were assessed at generation 3, to allow acclimatisation to the cage environment. After stabilising at generation 3, the frequency of both the RS and RR genotypes did not change significantly (ns p=0.24 and 0.77) between generations 3 and 9, whilst the frequency of SS declined (*p =0.04).*

Figure 3.15 The observed versus expected frequencies of each of the three genotypes in the population cages over time.



(a) The frequency of SS individuals is generally above that expected under Hardy Weinberg. (b) The frequency of RS individuals closely matches expectation, suggesting that they have no fitness disadvantage in the cages. (c) The frequency of RR individuals is significantly lower than expected throughout the course of the experiment. This shows that RR individuals show a phenotypic cost but that RS flies do not

Figure 3.16 Frequency of resistance over ten generations in the two sets of population cages with altered rates of empty.



The average percentage of resistant flies (RR and RS) over the first nine generations in cages emptied at 3, 5 and 7 days post-emergence of adult flies. Error bars indicate standard error, six repeats per data point.

The life history studies revealed that there is a difference in the rate of development of the RR and RS genotypes when compared with the SR and SS genotypes. The population cages establish each generation with a subset of flies that emerged within 7 days of the emergence of the first fly. The experimental design of the cages could therefore be selecting for one genotype over the others. To test this hypothesis cages were established using the same method but with flies for each generation collected at varying points post emergence. The results of these cages collected at five and three days post eclosion do not show the initial drop in the frequency of the *Accord*, which were seen when the flies were collected seven days after eclosion (figure 3.16). The *Accord* frequency increases in frequency over the nine generations of the cages and the trend is similar for all three sets of cages. These results indicate that the rate of empty of the cages does not have an effect on the frequency of the *Accord* element. In all three sets of cages the *Accord* appears to carry no cost.

The results from the population cages have several implications for the evolution and spread of *DDT-R* in natural populations. First, *DDT-R* mediated resistance to insecticides is dominant. Therefore flies that carry only one resistance allele (RS) can survive similar doses of DDT to flies that carry two (RR) and, as shown here, these RS flies do not carry any fitness cost. Combined with the continued selection for DDT resistance by other compounds to which *DDT-R* confers cross-resistance, the apparent lack of cost to resistance in the population cage studies may help explain why the frequency of *DDT-R* is currently approaching fixation in non-African populations of *D. melanogaster*. Second, as we know that the molecular basis of resistance is over-transcription of *Cyp6g1* and the corresponding over-expression of the insecticide degrading CYP6G1 cytochrome P450 enzyme, we can speculate as to the likely physiological reasons for the presence or absence of a cost. Thus flies only carrying one resistance allele clearly make enough enzyme to metabolise DDT but not to lose the unknown wild-type function of the *Cyp6g1* gene. Whereas flies carrying two copies of the resistance allele are at a competitive disadvantage in our population cages, possibly because wild type function of *Cyp6g1* is impaired. Alternatively the resource allocation towards DDT resistance is costly to the fly in the absence of selection and therefore the most advantageous situation is the heterozygous state.

3.3.3 Implications for resistance management

The key to managing resistance is to reduce selection pressure. High pesticide doses rapidly select for resistance. The Insecticide Resistance Action Committee (IRAC) recommends a number of resistance management guidelines to keep pesticides for crop pests and vectors working effectively and keep costs down (IRAC, 2006). The number of treatments and the concentration of treatments may be varied in practices considered to be good resistant management (McKenzie 1996). In theory, using low frequency doses of insecticide to increase survivorship of susceptibles can reduce selection for resistance.

One of the most commonly used resistance management strategies involves rotating or mixing products from different classes based on modes of action and where there are multiple applications per year, alternate products of different classes. This assumes a reduction in the frequency of the resistant genotype in the absence of the insecticide. If the resistant phenotypes show increased fitness however, it can be assumed that the frequency of resistant genotypes would increase under these conditions. Furthermore, the strategy assumes no cross-resistance to alternative compounds. *DDT-R* shows cross-resistance to a wide variety of compounds (Daborn, Yen et al. 2002) due to the broad spectrum of compounds that can be metabolised by *Cyp6g1*.

Alternative strategies that do not assume a reduced fitness of the resistant phenotype are important in trying to reduce the development of resistance in the field. The IRAC recommend that for crops, options for minimizing insecticide use include selecting early maturing or insect-resistant varieties, managing the crop for 'earliness'. In addition to chemical treatments, efficient cultural and biological control practices in pest control programmes can be used, in particular the careful selection of crop protection tools not only for cost and effectiveness but also for ability to maintain beneficial insects (IRAC, 2006). It is important that the pest or vector populations are monitored for resistance and the effectiveness of the control pesticide is monitored. In the event of a control failure that can be linked with resistance it is important that the pest is not re-sprayed with an insecticide from the same class.

3.3.4 Conclusions

Two individual approaches have been applied to determine the fitness effects of *Cyp6g1* overexpression resulting from the insertion of a transposable element, the *Accord* element within the 5' region of *Cyp6g1*. First, an extensive study of individual fitness traits, which gives an indication of differences in the strains' abilities to reproduce and survive and second, a population cage study, which allows the frequency of the *Accord* element to be recorded over a number of generations. The two methods give slightly differing results, with the life history experiments indicating a strong fitness advantage to the offspring of resistant females until the pupal stage. In the pupae and the adult, there is an apparent advantage to *Cyp6g1* overexpression regardless of whether this is inherited from the male or female parents. The population cages indicate that the heterozygous resistant individuals have a competitive advantage and maintain the *Accord* in the population, to the detriment of the homozygous resistant flies.

There are several explanations for this interesting difference in the performance of the homozygous resistant flies in the two experiments. The population cages test the overall performance of the genotypes under crowded conditions. Within the cages there is a high level of competition for food for larvae and adult flies. The fecundity of flies within crowded conditions showed the same trend as under uncrowded conditions with over two times the number of eggs laid by female RR, however the viabilities of the life stages and larval competition under crowded conditions with limited resources were not tested. The strains may show differential survival under more stressful conditions. Comparisons of studies using population cages and individual life history traits of methoprene mutants of *Drosophila* (Minkoff and Wilson 1992) lead to the conclusion that either a modest difference in a single life history trait can have appreciable effects or other fitness components they did not study may have an effect. The life history traits measured are not an exhaustive list and there are many other important traits to be measured. Sexually selected traits such as male competition and sperm competition are also important. These traits are often overlooked in life history studies but may have an important role in maintaining a beneficial, sexually selected phenotype within a population. Future studies should look at sexually selected traits to complement this study of naturally selected traits. The results

from both the life history and population cage work indicate that there is no cost associated with the carrying the resistant *DDT-R* allele. This is an interesting result and is in contrast to a number of other studies into the costs of resistance in other species and by other mechanisms of resistance. Much further work is required to confirm that the apparent fitness benefit observed in these experiments is due to *Cyp6g1* over expression.

CHAPTER 4

The role of *Cyp6g1* in reproduction

4.1. INTRODUCTION

4.1.1 Factors affecting *Drosophila* fecundity

The previous chapter identified a possible reproductive fitness benefit to the resistant flies. Due to the complexity of development of growth and reproduction in insects this chapter will focus on understanding the difference in fecundity of resistant and susceptible flies demonstrated in the previous chapter. The fecundity of a female *Drosophila* may be affected by the rate of the production of embryos or by the interactions between the male and female on mating which affects the rate of egg lay. Short-range pheromones are thought to affect female oviposition without male mating (Hoffmann and Harshman 1985). In addition females that mate at high frequencies suffer fitness costs (reduced longevity and reproductive success) as a result of the actions of male seminal fluid accessory gland proteins (Acps) (Chapman, Liddle et al. 1995). Acps mediate a variety of effects that benefit males including the stimulation of female egg production, reduction of female receptivity, ensuring effective sperm storage, and promotion of male success in sperm competition (Wigby and Chapman 2005). One Acp, the sex peptide (SP) is thought to stimulate egg production by causing the release of juvenile hormone (JH) from the corpora allata (CA) (Moshitzky, Fleischmann et al. 1996). This release stimulates oocyte progression in the ovary (Soller, Bownes et al. 1999).

4.1.2 Cytochrome P450 involvement in reproduction

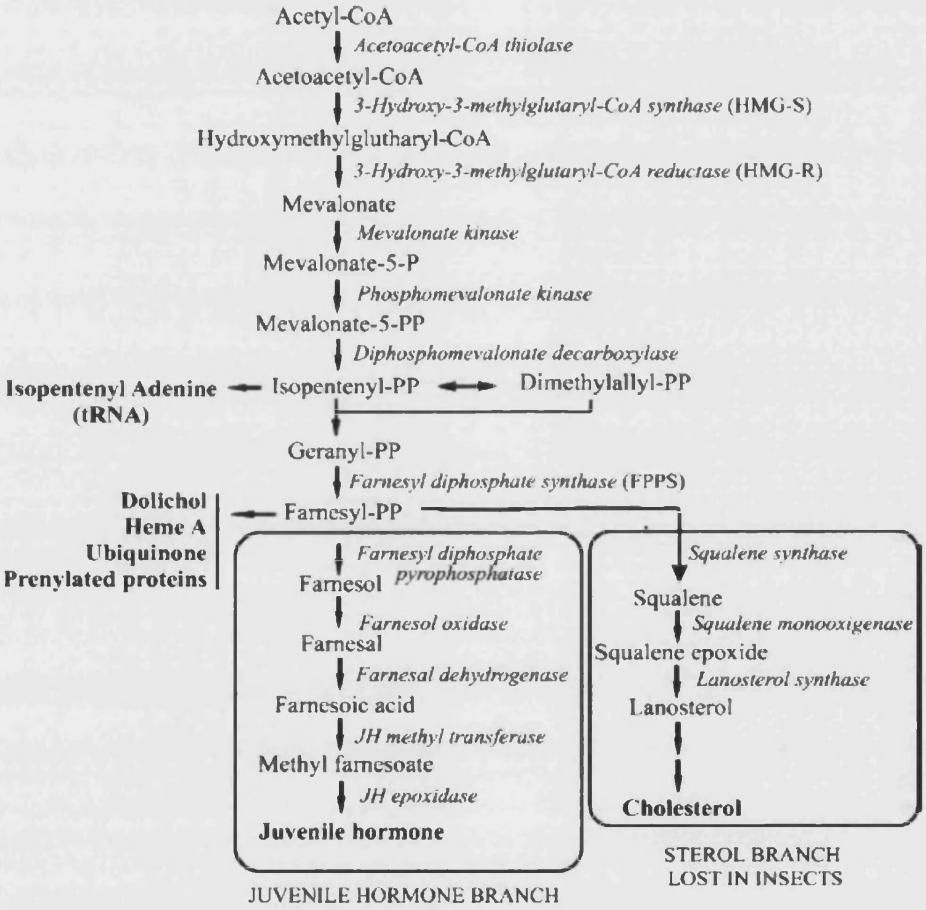
A study of male reproductive success under competitive conditions (MCRS) identified a number of genes with transcriptional variation significantly associated with male fitness variation. One of these genes was *Cyp6g1*. The study demonstrated that low MCRS is associated with higher expression of *Cyp6g1*, however the presence of the *Accord* in these strains was not taken into account. The study indicated *Cyp6g1* expression in males may introduce a cost, however looking at a single trait does not help in understanding the overall effect of *Cyp6g1* over expression on the fitness of flies.

Cytochrome P450 enzymes have been shown to involved in hormone synthesis and degradation reproduction in insects. Insect moulting is triggered by pulses of ecdysone (20E) released from the prothoracic gland in response to a neuropeptide signal from the brain (Chang 1993). Ecdysteroids have been implicated in regulating oogenesis (Buszczak, Freeman et al. 1999). 20-hydroxyecdysone (20E) biosynthesis is thought to involve a number of cytochrome P450 catalyzed hydroxylations of cholesterol (Chavez, Marques et al. 2000). The wild-type genes of members of the Halloween family of embryonic lethals, *disembodied* (*dib*), *shade* (*shd*), and *shadow (sad),* code for mitochondrial cytochromes P450 that mediate the last two hydroxylation reactions in the ecdysteroidogenic pathway in *Drosophila* (Warren, Petryk et al. 2002). The expression of *sad* and *dib* is concentrated within the individual segments of the developing epidermis when there is a surge of ecdysteroid midway through embryogenesis, before the development of the ring gland suggesting that the embryonic epidermis is a site of ecdysteroid biosynthesis (Warren, Petryk et al. 2002). The *Drosophila* homologue of the Cytochrome P450, *Cyp306a1*, has been shown to be disrupted in the *phantom (phm)* mutant, another member of the Halloween group. The associated morphological defects and the decreased expression of ecdysone-inducible genes in the *phm* mutant suggests that the *phm* mutant produces a reduced titre of ecdysone (Niwa, Matsuda et al. 2004).

cDNA encoding the cytochrome P450, CYP4C7, was isolated from a cDNA library of the CA from reproductively active *Diploptera punctata* (Sutherland, Unnithan et al. 1998). CYP4C7 expressed in a heterologous system metabolizes JH III and JH precursors to their 12-trans-hydroxy metabolites. The timing of CYP4C7 gene expression and its catalytic activity suggests that it is involved in the catabolism of sesquiterpenoids within the CA during the developmental stages, when an intrinsic repression of JH synthesis is observed (Sutherland, Unnithan et al. 1998). The authors showed that in *D. punctata,* JH excess at the end of the cycle resulted in the inhibition of ovulation and abortion of early embryos. CYP4C7 expression was shown to be high in postvitellogenic insects, when a reduction in JH titre is critical for allowing oviposition and preventing abortion, as suggested by data on topical application of JH III (Sutherland, Unnithan et al. 1998). In addition the cytochrome P450, CYP15A1 was identified from expressed sequence tags (ESTs) from the corpora allata of the cockroach

Diploptera punctata (Helvig, Koener et al. 2004). This cytochrome P450 is thought to be the epoxidase of Methyl farnesoate, the precursor of JH (figure

Figure 4.1 The juvenile hormone biosynthesis pathway



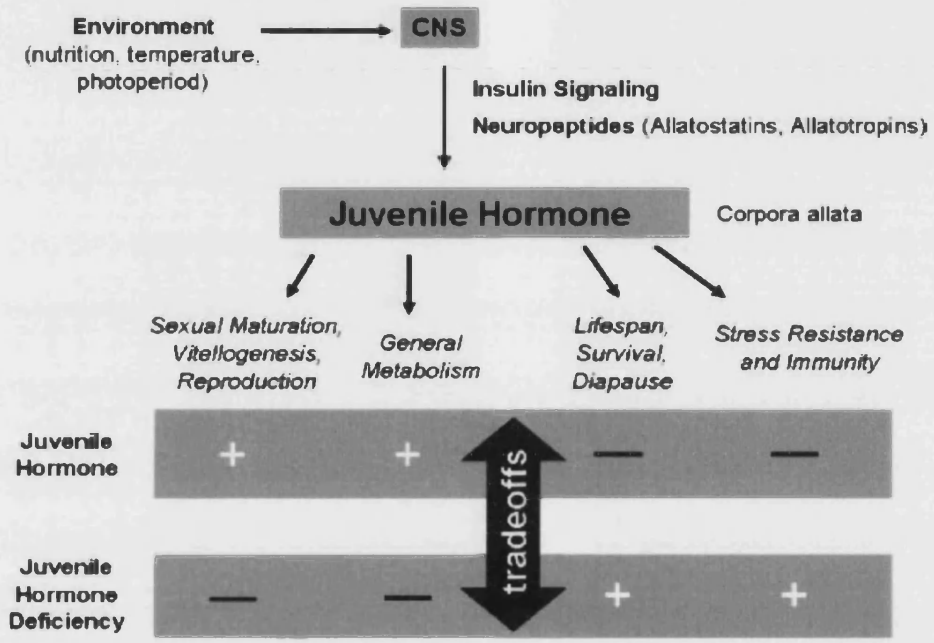
Flux diagram of the mevalonate pathway and JH biosynthesis in insects. Other important final products and the final steps leading to cholesterol in vertebrates have been lost in insects (taken from Belles, Martin et al. 2005).

4.1). CYP15A1 activity was found to be highly specific. The enzyme is thought to be selectively expressed in the CA, no transcript was detected in either brain or fat body. In *M. domestica*, CYP6A1 is expressed in the gut and fat body is capable of the metabolising the isomers of methyl farnesoate to the 10,11-epoxides (Andersen, Walding et al. 1997).

4.1.3 Juvenile hormone

Modelling of *Cyp6g1* based on the protein sequence and other insect Cytochrome P450 structures shows that JH may fit into the active site of the enzyme (Jean van den Elsen, personal comment). Juvenile hormone synthesis is affected by both internal (e.g. hormonal, genetic) and external (e.g. temperature, nutrition) factors, to regulate and coordinate the expression of entire gene batteries, and to simultaneously affect multiple phenotypes (Flatt, Tu et al. 2005). JH affects a wide range of processes and traits in *Drosophila* development and life history, including metamorphosis, behaviour, reproduction, diapause, stress resistance and aging (Flatt, Tu et al. 2005). The role in moulting, important in the activity of JHAs, is described in Chapter 1. A further role of JH is in oogenesis (figure 4.2). JH is synthesised in the corpora allata (CA), located within the ring gland, the major endocrine organ of *Drosophila* and is degraded predominantly by esterases and epoxide hydrolases (Wilson 2004). In *Drosophila* and higher Diptera, the JH methyl 6,7;10,11-bisepoxyfarnesoate (JHB3) is the predominant JH produced by the ring gland and isolated corpora allata (Richard, Applebaum et al. 1989). The biosynthesis of JH during the reproductive cycle of the cockroach *Diploptera punctata* has been extensively characterized (Sutherland, Unnithan et al. 1998). In *D. punctata* adult females JH synthesis is regulated by humoral factors and by innervation from neurosecretory cells in the brain (Sutherland, Unnithan et al. 1998). Production of JH by the CA increases 10-fold to reach a peak 5 days after adult emergence and mating, and this peak corresponds to the peak of vitellogenesis. Synthesis is then rapidly repressed and remains low from deposition of the eggs into the brood sac through pregnancy (Tobe, Ruegg et al. 1985).

Figure 4.2 The pleiotrophic action of juvenile hormone in insect life history



Juvenile hormone synthesis and signalling are regulated by insulin signalling and neuropeptides. JH coregulates metabolism, reproduction, stress resistance immune function and lifespan (taken from Flatt, Tu et al. 2005).

The process of vitellogenesis requires the synthesis of the yolk protein (YP) precursors, the vitellogenic proteins e.g. vitellogenin in the fat body of the insect, their elease into the hemolymph and subsequent uptake by the growing oocytes against a concentration gradient (Engelmann 2002). In *Drosophila*, vitellogenin is synthesized in the fat body and ovaries (Isaac and Bownes 1982). One theory is that in Drosophila JH initiates only the early stages of vitellogenesis and ecdysteroid synthesis in the ovaries, while the main role in the direct control of oogenesis is played by 20E, which stimulates the synthesis of YPs inthe fat body and their uptake by the oocytes (Richard, Jones et al. 2001). An alternative hypoothesis however, proposes that the development of vitellogenic oocytes, including both synthesis of YPs in follicular cells and their uptake by the oocytes, is stimulated by JH, and that 20E controls previtellogenic stages of oocyte development (Soller, Bownes et al. 1999).

Increased JH levels may be negatively associated with lifespan in some insects. The removal of the CA in monarch butterfly results in a 100% increase in lifespan (Herman and Tatar 2001). JH deficiency, which results from a mutation in the gene *insulin-like receptor* (*InR*), is sufficient to extend life-span in *Drosophila.*

4.1.4 Aims of chapter

The previous chapter identified a possible reproductive fitness benefit to the resistant flies. In particular the insertion of the *Accord* transposon is associated with an increase in the fecundity of resistant individuals. This has important implications in resistance management and in the understanding of the native role of the Cytochrome P450, *Cyp6g1*, which confers resistance. The aims of this chapter are to investigate the potential role of *Cyp6g1* in reproduction in *Drosophila melanogaster*. The chapter will focus mainly on studying the effect of *Cyp6g1* expression on the fecundity of *Drosophila*, in an attempt to understand the results presented in Chapter 3. The UAS GAL4 expresssion system will be used to over express and knock down *Cyp6g1* expression in order to determine the effect of manipulating *Cyp6g1* expression on the fecundity of female flies. The effect of juvenile hormone on the fecundity of resistant and susceptible flies will be investigated. Quantitative real time PCR will be used to quantify *Cyp6g1*

expression during development. Finally, GFP expression can be driven with the 5' of the resistant and susceptible alleles, as shown in Chapter 2. This technique will allow the study of *Cyp6g1* expression in the reproductive tissues and allows a comparison of *Cyp6g1* expression in the reproductive tissues of resistant and susceptible flies.

4.2. MATERIALS AND METHODS

4.2.1 Strains used

The laboratory standard strain Canton-S, obtained from the Bloomington Stock Centre was used as the wild type DDT susceptible strain (*SS*). The backcrossed strain (*RR*), described in the previous chapter was used as the DDT resistant strain. *UAS-6g1* strains were constructed by Phillip Daborn (University of Melbourne). The *UAS-RNAi* strain was obtained from Bruno Lemaitre (Centre de Génétique Moléculaire, CNRS). Paired strains were obtained from Markus Noll (University of Zürich). Son of Tudor, Oregon-R and GAL4 driver strains were obtained from the Bloomington Stock Centre.

4.2.2 *Cyp6g1* involvement in fecundity

4.2.2.1 Virgin female egg laying

To determine if the difference in the fecundity is due to differential responses from the female post mating the fecundity of virgin females was recorded. Virgin females from the lines Canton-S, the backcrossed strain, Oregon-R and Hikone-R were collected and kept at a density of 20 flies per vial for 2 days. A single virgin female was introduced into each chamber of the manifolds. A total of 20 females per line were used. Manifolds were incubated at 25°C under constant light. The agar plates changed daily and the eggs laid were counted every 24 h for 5 days. A total of 60 females per line were studied. Data were recorded in Microsoft Excel and statistical analyses performed in MINITAB 12.0. Data were analysed using analysis of variance (ANOVA) and Tukey's post hoc test.

4.2.2.2 *Cyp6g1* over expression and knockdown

The UAS/GAL4 expression system was used to study the effect of *Cyp6g1* over expression and knockdown on fecundity. To over express *Cyp6g1*, the strain *Tub-GAL4* was crossed with the *UAS-6g1* strain. The flies resulting from this

cross have been shown to express around 10 times more *Cyp6g1* than the control flies. Virgin females from the *UAS-6g1* strain were crossed to males of the *Tub-Gal4* strain and were allowed to lay for 24 hours in a food vial and then removed. The *UAS-6g1* flies do not lay large numbers of eggs and so there was no need for measures to prevent crowding in the food vials. The food vials were stored at 25 ºC until the progeny emerged. Knockdown of *Cyp6g1* was performed as described in Chapter 2.

Virgin females from the knockdown and over expression crosses were collected and their fecundity was measured using the egg collecting manifolds. Manifolds were incubated at 25 ºC under constant light and eggs counted every 24 h for 5 days. A total of 60 females per line were studied. Data were recorded in Microsoft Excel and statistical analyses performed in MINITAB 12.0. Data were analysed using analysis of variance (ANOVA) and Tukey's post hoc test.

4.2.2.3 Effect of juvenile hormone on fecundity

Virgin female flies of the strains Canton-S and Hikone-R were collected at 3-5 days old. Juvenile hormone III (Sigma) was diluted in acetone and 200 µl added to 23 ml glass scintillation vials. The vials were rolled to evenly coat the inside of the vial with the juvenile hormone. Vials were rolled at concentrations of 10.0, 1.0, 0.1 µl Juvenile hormone per vial. 30 flies were added to each vial and the vials were bunged with cotton wool soaked in a 5% (w/v) solution of sucrose. Six vials were prepared per concentration. Vials were stored at room temperature overnight. After 8 hours of juvenile hormone exposure the flies were removed and individually added to a food vial. Half the females were added to a food vial alone, the other half with a single male of the strain Oregon-R. The flies were moved to new food vials daily and the number of eggs laid by each female was recorded. Data were analysed in Microsoft Excel and MINITAB 12.0. ANOVAs followed by Tukey's post hoc test were performed on the strains to determine whether the number of eggs laid by individuals of each strain varied according to JH exposure.

4.2.3 Expression of *Cyp6g1* in reproductive tissues

4.2.3.1 GFP expression patterns

The UAS/Gal4 expression system was used as described in Chapter 2 to study *Cyp6g1* expression in reproductive tissues. Females were collected at 5 days old and dissected in Ringers dissection media (Appendix A3). The reproductive organs were separated under a dissecting microscope (Zeiss) and fixed with ProLong Antifade (Invitrogen) following manufacturers instructions and mounted on slides for viewing. Slides were viewed under the confocal microscope (Zeiss) and images viewed with the Zeiss LSM meta browser software.

4.2.3.2 Son of Tudor/Paired

Son of Tudor *(tud^1)* is a *Drosophila* strain that produces accessory gland proteins (Kalb, DiBenedetto et al. 1993) but no sperm. When mated to a female of another strain, the resulting cross produces embryos but these are not viable. Paired strains (*prd*) lack accessory glands so produce sperm but no accessory gland proteins (Xue and Noll 2000). Females mated to paired strains lay no fertile eggs. A reciprocal cross can be used to restore the function of the sperm, i.e. if a female is mated to a Tudor then Paired male, she will lay fertile eggs. These strains were crossed to the 5'HR-Gal4/UAS-GFP females to look at the GFP expression in females that were expressing GFP under the control of the 5' of *Cyp6g1* to see whether the GFP expression patterns varied with the presence of sperm only, accessory gland proteins only or the presence of both together.

Females mated to paired and Son of Tudor males were dissected in Ringers dissection media and the reproductive tissues examined for GFP expression. Females were collected at 5 days old and dissected and fixed as described above. In addition, embryos were isolated from the female egg sac. Virgin females were allowed to lay on egg laying agar and embryos collected and fixed in ProLong Antifade as described before. Slides were viewed under a fluorescence microscope (Leica) and images were viewed with Microsoft Photoshop.

4.2.4 Expression of *Cyp6g1* in sperm

4.2.4.1 GFP expression in sperm

To determine if expression of *Cyp6g1* in resistant construct males was in sperm, the sperm had to be isolated from the rest of the reproductive tissue. Males were collected as virgins and held in food vials for 20 days to allow an increase in the amount of sperm in the testes. Seminal vesicles were dissected from males and burst with a dissecting needle to tease out the sperm. Sperm were rinsed in Ringers dissecting buffer and fixed and mounted as described in Chapter 2.

4.2.4.2 GFP antibody

To confirm that GFP is expressed in sperm and that the observed expression in previous study was not autofluoresence, the sperm was stained with a Goat polyclonal antibody raised to GFP (Abcam). The sperm were extracted from the seminal vesicles as previously described, washed in 1x Phosphate Buffed Saline (PBS, Oxoid) and fixed in 4% (w/v) paraformaldehyde for 20 min at room temperature. Following this the sperm were washed three times in 1 x PBS and permeabilised in 0.2% Triton X-100 in PBS for 10-15 min. After three more washes sperm were incubated in 0.1% Bovine Serum Albumin (BSA, Sigma) in PBS on a rocking platform. The sperm were incubated in a 1/5000 dilution of the primary antibody in 0.1% BSA in PBS for 90 min at room temperature. The sperm were then washed three times in 1 x PBS and twice in double distilled H_2O and then were mounted on a microscope slide for viewing. Slides were viewed under a fluorescence microscope (Leica) and images were viewed with MS Photoshop.

4.2.4.3 Western blotting of sperm with *Cyp6g1* antibody

A CYP6G1 polyclonal antibody was raised in rabbits against a CYP6G1-MBP fusion protein by Sam Boundy (University of Bath). Sperm were extracted as previously described and boiled in 1 x SDS (sodium dodecyl sulphate) loading

buffer for 5 minutes at 100°C. The quantity of protein in each sample was measured using a EZQ Protein Quantitation Kit (Invitrogen) following manufacturers instructions. The samples were run on 2 identical 12% SDS PAGE (polyacrylamide gel electrophoresis) gels for 2 hours at 100 V. 5 µg protein was loaded into each well. Control wells were also set up with containing 5 µg total protein from Hikone-R and cloned, purified CYP6G1 (kindly donated by Robert Jones, University of Bath). The Hikone-R total fly preparation was prepared by crushing 20-30 whole Hikone-R flies in 50 µl Ringers dissection buffer, adding 200µl 1x SDS loading buffer and boiled for 5 min at 100°C. One gel was then incubated in Western Transfer Buffer (36.4g Tris base, 150 g Glycine, 10 ml 20% SDS and ddH_2O to 1 l) for 1 hour on a shaking platform. The second gel was stained with Coomassie blue stain for 1 hour before 3 x 5 min incubation in destain buffer (450ml Methanol, 100ml acetic acid made up to 1 l with ddH20). The gel was incubated in Gel Drying Solution (50 ml acetic acid, 150 ml Methanol, 25 ml glycerol made up to 500 ml ddH_20) for 20 min. The gel was then laid on cellophane and dried using the Mini Gel drying system (Invitrogen) overnight.

The first gel, used for the Western blot, was blotted onto a nitrocellulose membrane (Immun-Blot PVDF Membrane, Bio-Rad) that had been pre-soaked in methanol. 6 pieces of 3 mm chromatography paper were cut to the size of the gel (8 cm x 9 cm). These were soaked briefly in western buffer and 3 pieces placed on the trans blotter. The membrane was added on top of this followed by the gel then 3 more pieces of blotting paper. Air bubbles were removed by rolling a 50 ml tube over the top layer of blotting paper being careful not to dislodge the gel or membrane. The membrane was blotted using a using a trans-blot semi dry transfer cell (BioRad) running at 15V for 15 min. The membrane was stored overnight at 4°C in 5% skimmed milk powder (Marvel) dissolved in Washing Solution (0.5% Tween 20 in 1x PBS). The membrane was washed for 5 min in Washing Buffer to wash off the milk. The primary antibody was then added in 20 ml Washing Buffer containing 5% milk powder and shaken at room temperature for 2 h, a 1 in 1/1000 dilution of primary antibody was found to give the best western results. The membrane was then washed 4 times for 20 min in Washing Buffer and the secondary antibody added to 20 ml Washing Buffer containing 5% milk powder. The secondary antibody used was an anti-rabbit alkaline

phosphotase (AP) antibody (Sigma). A dilution of 1/10,000 of the primary antibody gave the best results. The membrane was then washed three times for 20 min in Washing Buffer before developing. The bands were visualised using a Nitro blue tetrazolium chloride/5'Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) developing solution (Roche). 175 µl of the NBT/BCIP solution was added to 10 ml of developing solution (100 mM Tris HCl, 10 mM $NaCl_2$, 5 mM $MgCl_2$ pH 9.5). 5 ml of this solution was poured over the membrane and the membrane was observed for bands. Once the bands were strongly visible, the membrane was washed with distilled water to stop the reaction.

On initial blots, very high levels of non-specific binding of the antibody resulted in multiple bands on the blot. To reduce the non-specific binding the antibody was purified by covalent binding by Robert Jones (University of Bath). The His-tag purified CYP6G1, refolded in arginine, was covalently bound to an NHS-activated column, allowing for anti-CYP6G1 antibodies to be purified from a polyclonal anti-CYP6G1-MBP (CYP6G1-maltose binding protein fusion) rabbit serum. Firstly, arginine was removed from the buffer and the protein was exchanged into Ligand Coupling buffer (0.2M NaHCO3, 0.5M NaCl, pH 8.3) by concentration and resuspension in a 10,000 MWCO centrifuge concentrator. A 1 ml HiTrap NHS-activated HP column (GE Healthcare) was prepared following the manufacturers directions. The column was injected with the ligand solution and serially washed with Buffer A (0.5 M ethanolamine, 0.5M NaCl, pH 8.3) and Buffer B (0.1M acetate, 0.5 M NaCl, pH 4) to deactivate excess active groups. 4 ml of antibody serum was diluted in 20 ml of phosphate buffered saline (PBS). The dilution was slowly (~1ml/min) passed through the column by syringe, and the flow through was collected. The column was washed with 10 ml PBS and 10 µl samples were collected at 5 ml and 10 ml to examine by SDS-PAGE. Bound antibodies were eluted with 8mlof 0.2 M glycine pH 2.5, collected in 1ml fractions and immediately restored to pH 7 with 1 M Tris pH 8. 10 µl samples of the eluted fractions were analysed by SDS-PAGE, and used for Western blots of recombinant protein and *Cyp6g1*-expressing *D. melanogaster*. The antibodies were stored at 4°C.

An antibody adsorption method was performed by Robert Jones (University of Bath) in addition to the purification of antibodies using the target protein covalently bound to a column (University of Bath). A pellet of Rosetta cells

expressing CYP6G1 was resuspended to an OD 600 of 2.0 and from this 2 x 1 ml was pelleted. Each pellet was resuspended in 300 µl of 1 x SDS loading buffer, heated at 100°C and loaded onto an SDS gel prepared with a single well spanning the entire gel. The SDS-PAGE gel was transferred onto a nitrocellulose filter at 100 V for 1 h and the filter was blocked with PBS, 0.1% Tween and 5% milk powder for 1h. 2 ml of serum was added and kept at 4°C O/N. The antibody solution was discarded and the filter washed with 0.15 M NaCl, then PBS. A scalpel was used to excise a strip of~1cm from each side of the filter. The strips were incubated in 10 ml of 1:1000 PBS-diluted Horseradish Peroxidase (HRP)-conjugated anti-rabbit antibody for 1-2 h. The strips were stained with NBT/BCIP (Roche) method to visualise the bound, antibodies. The strips were re-aligned with the major part of the filter and the band corresponding to CYP6G1 was excised. The CYP6G1 specific antibodies were eluted with 250 µl 0.2 M glycine pH 2. 1 mM EDTA. The eluate was collected into microcentrifuge tubes and neutralised and stored as above, with 0.1 volumes of 10 x PBS and 0.02% sodium azide.

4.3. RESULTS AND DISCUSSION

4.3.1 *Cyp6g1* involvement in fecundity

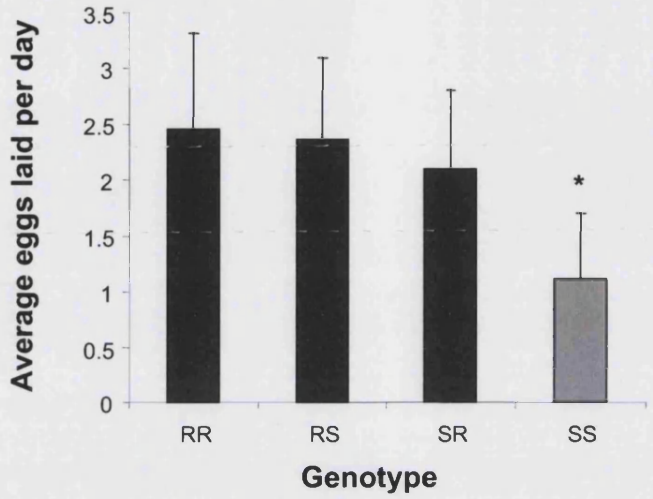
4.3.1.1 Virgin female fecundity

The number of eggs laid by virgin females over a five-day period was recorded. The number of eggs laid by the *RR, RS* and *SR* genotypes was significantly higher than the number of eggs laid by the *SS* flies (F= 12.35 p= 0.03). The number of eggs laid by the virgin flies was highly variable as indicated by the error bars (figure 4.3). The results were statistically significant at the 5% level however. Mating induces an increase in the egg laying rate, compared with virgin females (Kubli 2003). The number of unfertilised eggs laid by virgin flies is a useful indication of the number of eggs produced by the fly when there are no influences from the male. These results indicate that the higher fecundity observed in *RR, RS* and *SR* females, when compared with the SS genotype is due to pre-mating factors. The following work will attempt to show a link between *Cyp6g1* expression and fecundity.

4.3.1.2 *Cyp6g1* over expression effect on fecundity

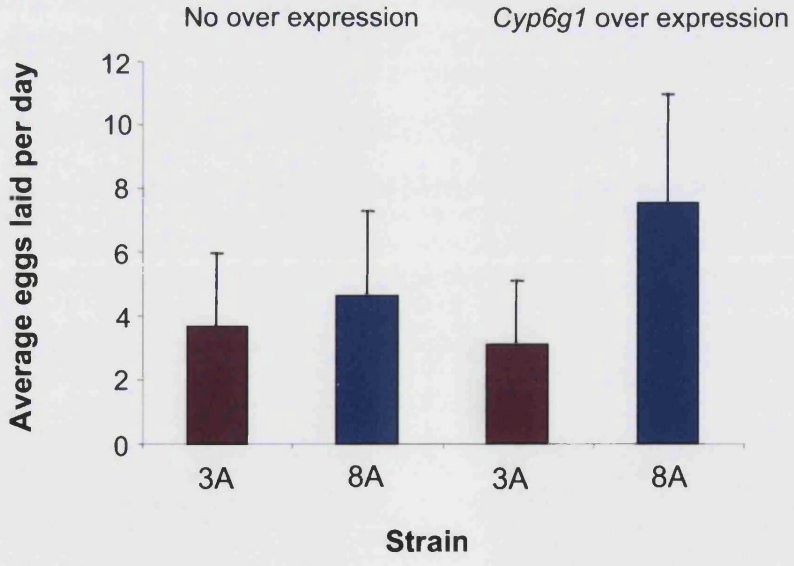
The UAS GAL4 expression system was used to constitutively over express *Cyp6g1* using a tubulin-GAL4 driver strain. The number of eggs laid over a five-day period was recorded for two different strains, 8A and 3A, produced by independent insertions of the UAS construct (figure 4.4). The control flies of the strain 3A, which contain the UAS construct, but not the GAL4 driver laid on average a slightly higher number of eggs than the flies containing both the UAS and GAL4 constructs. This result was not statistically significant however (T= 6.73 p= 0.68). The second strain, 8A, showed higher egg lay in the flies containing both the UAS and GAL4 constructs than the flies containing just the UAS construct but this was not significant (T= 8.92 p= 0.19). On balance it appears that when *Cyp6g1* is over expressed using the UAS GAL4 expression system, there is no effect on the fecundity of the flies.

Figure 4.3 Fecundity of virgin females of the different *DDT-R* genotypes over a five day egg laying period



*The fecundity of each genotype is plotted with standard error bars, a * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons).*

Figure 4.4 Fecundity of transgenic females over expressing *Cyp6g1* over a five day egg laying period



The fecundity of two independently transformed strains, 3A, plotted in pink and 5A plotted in blue are plotted with standard error bars. The differences between the over expressing and non over expressing forms of the strains are not significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons).

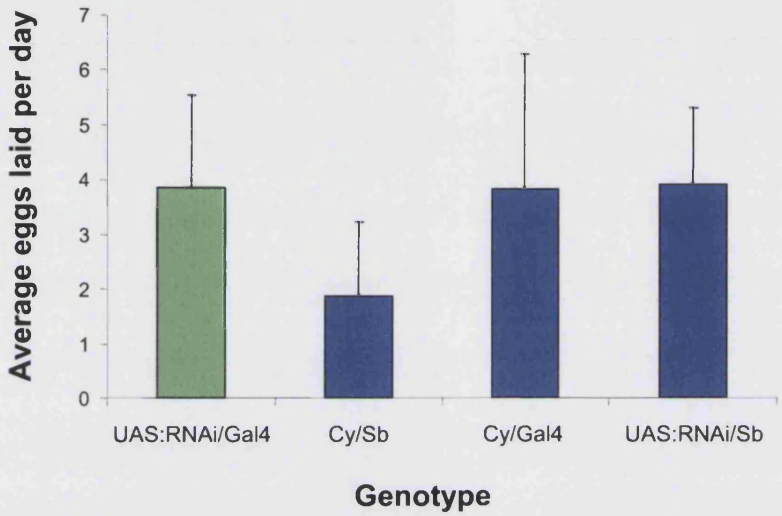
This is in contrast to the results with the backcrossed flies, which indicated that flies carrying the *Accord* element and therefore overexpressing *Cyp6g1* show higher fecundity, compared with those not carrying the *Accord* element. There are a number of possible explanations. First the use of transgenics may not be successful when studying life history traits. The fecundity of the transgenic flies is much lower than expected for young *Drosophila* and some flies did not lay any eggs over the period of the study. This indicated that the effect of *Cyp6g1* over expression may be masked by the reduced fitness in the transgenic flies. The UAS GAL4 system has been successfully applied to a number of life history studies; however the position of the insertion and the background genome of the flies may have affected the results. Second, the results may indicate that the over expression of *Cyp6g1* does not result in increased fecundity. The insertion of the *Accord* may result in the upregulation of another unidentified gene, which is resulting in the increased fecundity. Alternatively the selection for DDT resistance during the backcrossing may have selected for another closely linked gene, although with 20 generations of backcrossing the background genome would be virtually identical.

One further method which could be applied to see whether increasing *Cyp6g1* expression affects fecundity would be to induce *Cyp6g1* over expression with the compound phenobarbital (see Chapter 2) and measure the fecundity. The use of this compound would induce the over expression of a number of cytochrome P450s in the fly and so would not confirm that the over expression of Cyp6g1 alone is sufficient to confer increased fecundity, however the induction could be performed with strains which do not show suppressed fecundity, unlike the transgenic experiments which appear to have an effect on the overall fitness of the flies.

4.3.1.3 Effect of *Cyp6g1* knockdown on fecundity

The UAS GAL4 expression system was used to constitutively knock down *Cyp6g1* using a tubulin-GAL4 driver strain (see Chapter 2 for details). The number of eggs laid over a five-day period was recorded (figure 4.5). The RNAi flies, which contain both the UAS and GAL4 constructs, are indicated in green.

Figure 4.5 Fecundity of transgenic females with *Cyp6g1* knocked down over a five day egg laying period



The fecundity of each genotype is plotted with standard error bars. The RNAi knockout is plotted in green, the RNAi controls are plotted in blue. There were no significant differences between the genotypes at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons).

The control genotypes, which do not contain both the UAS and GAL4 constructs necessary for the RNAi phenotype, are coloured in blue. There was no statistically significant difference in the number of eggs laid by the four different genotypes (T= 15.34 p= 0.069).

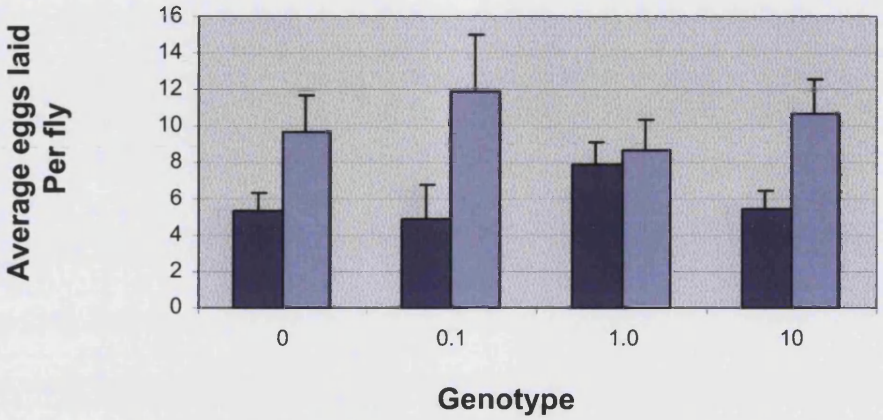
The previous use of this RNAi strain in Chapter 2 showed that the effect of knocking down *Cyp6g1* has little effect on DDT resistance due to the low expression of *Cyp6g1* in the control flies. It would be expected that the knockdown of *Cyp6g1* would not have noticeable effect on fecundity. It is important to note that although the knockdown of *Cyp6g1* does not appear to reduce the fecundity the fecundity of the flies is very low, as was also observed with the transgenic strains over expressing *Cyp6g1*. By inserting or crossing the UAS construct into a strain expressing greater levels of *Cyp6g1*, there may be a difference in the fecundity when *Cyp6g1* expression is knocked down.

4.3.1.4 Effect of juvenile hormone on fecundity

Modelling of *Cyp6g1* by Jean van den Elsen (University of Bath) has shown the interaction between active site of *Cyp6g1* and the broad range of substrates, which have been shown to be metabolised by *Cyp6g1* (personal comment). From this it appears that Juvenile Hormone fits into the active site of *Cyp6g1*. JHIII was applied to the flies and the fecundity of both virgin and mated female flies was recorded.

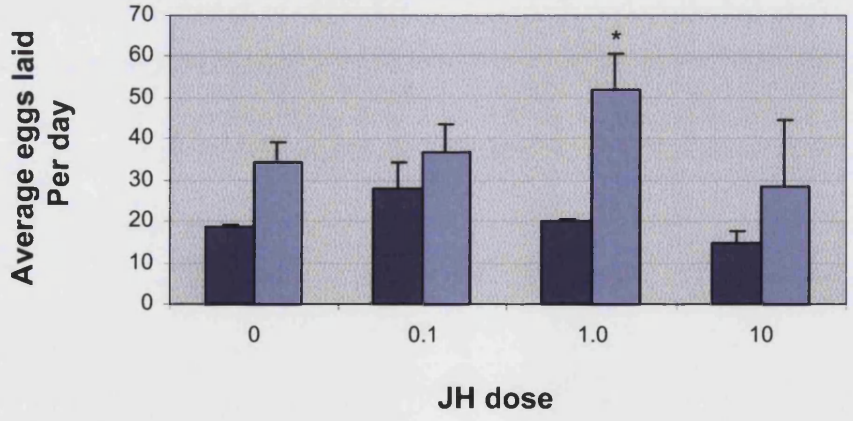
The results show that the application of JHIII to virgin female flies does not significantly change the number of eggs laid by either RR or SS flies at doses of 0.1, 1.0 or 10.0 µg/ml (F= 22.63, p= 0.091)when compared with no juvenile hormone exposure (figure 4.6). The fecundity of the RR flies was higher than the fecundity of the SS flies as seen in previous experiments. When JHIII was applied to mated female flies there was no significant change the number of eggs laid by either RR or SS flies at doses of 0.1, or 10.0 µg/ml when compared with no JH exposure (figure 4.7), however RR flies exposed to 1.0 µg/ml laid significantly more eggs (F= 12.88, p= 0.02). This result is confusing since JH exposure at a higher dose did not have a significant effect on fecundity. The

Figure 4.6 Fecundity of virgin females of different *DDT-R* genotypes over a five day egg laying period after exposure to juvenile hormone



*The fecundity of the RR (pale blue) and SS (dark blue) genotypes over the five day period are plotted with standard error bars, a * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons).*

Figure 4.7 Fecundity of mated females of different *DDT-R* genotypes over a five day egg laying period after exposure to juvenile hormone



*The average daily fecundity of the RR (pale blue) and SS (dark blue) genotypes are plotted with standard error bars, a * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons).*

results may indicate that JH has an effect on the fecundity of flies over expressing *Cyp6g1*, however further study is required to look at the effect of JH exposure on flies over expressing *Cyp6g1*. It is possible that the higher fecundity observed in mated females at one specific dose of JH exposure is an anomaly, an earlier preliminary experiment resulted in a drop in fecundity with JH exposure.

The lack of any convincing effect of JH on fecundity under laboratory conditions may be due to the application method of the JHIII. An alternative method of JH application is to apply the hormone to individual flies (Rauschenbach, Gruntenko et al. 1996). This method would be advantageous in that the direct application of the hormone would ensure a more consistent exposure between individual flies, it would however limit the population size that could be sampled. An alternative method of JH application would be to add the compound to the media. The amount of JH in flies can be quantified by LC-MS and this would provide a useful measure of the effectiveness of JH application as well as quantifying JH within the resistant and susceptible flies. Finally the use of the JHA methoprene may be preferable since the compound has been developed for its stability and may prolong JH exposure in the fly, increasing the effect on resistant or susceptible flies.

In addition the effect of JHIII application on *Drosophila* under stressful conditions would be important. Adult insects respond to unfavourable environmental conditions such e.g. high and low temperature, mechanical and chemical irritants and high density, by a neurohormonal stress reaction involving the metabolism of JH, dopamine (DA), octopamine (OA) and ecdysteroids (Gruntenko, Chentsova et al. 2003). The *D. melanogaster* strain ap^{56f} has increased levels of JH degradation. In this strain flies of both sexes have decreased viability under normal conditions, under heat stress, ap^{56f} females showed decreased survival whereas the males show survival equal to that of wild-type (Gruntenko, Chentsova et al. 2003).

The effect of increased JH on fecundity is complicated since treating wild type *Drosophila* with the JHA methoprene results in a prolonged developmental time, reduced body size at ecolsion and shortened adult lifespan (Flatt, Tu et al. 2005)

potentially reducing the lifetime output of a female. However methoprene application also increases early fecundity (Flatt, Tu et al. 2005) so it appears JH is regulating the trade off between the various life history factors.

4.3.2 Expression of *Cyp6g1* over the lifecycle of the fly

To study the expression of *Cyp6g1* over the lifecycle of the fly in the genotypes RR, RS, SR and SS, the amount of mRNA was quantified relative to the amount of mRNA of the ribosomal protein RP49.

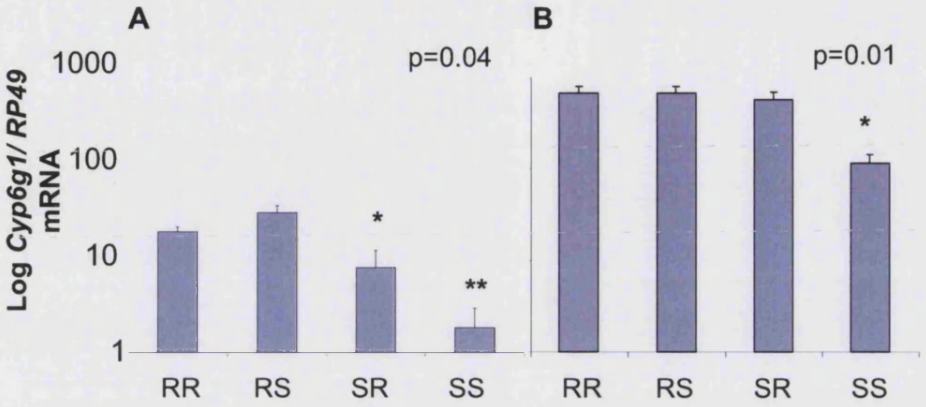
4.3.2.1 Quantity of mRNA in early and late embryos

Low levels of *Cyp6g1* expression were detected in 3-hour embryos of all genotypes (figure 4.8). The expression was highest in RR and RS embryos, the detected *Cyp6g1* in SR embryos was lower than in RR and RS and the amount of *Cyp6g1* detected in SS was much lower than RR, RS and SR ($F=23.61$ $p=0.042$). The presence of low levels of *Cyp6g1* at this early stage of development indicates that there may be maternal transmission of *Cyp6g1* mRNA. The higher levels of detected *Cyp6g1* mRNA in RR and RS compared with SR and SS genotypes is a further indication that there is maternal transmission of *Cyp6g1* since expression of *Cyp6g1*. The higher level of *Cyp6g1* detected in the SR compared with the SS however, indicates that there is some transcription occurring in the embryo at this early stage.

Quantification of the amount of *Cyp6g1* transcript is in late embryos (figure 4.8) indicates there is no significant difference between RR, RS and SR genotypes, whilst the amount of transcript is significantly lower in SS flies ($F=48.2$, $p=0.01$). The quantity of *Cyp6g1* mRNA relative to *RP49* is 80 fold higher indicating active levels transcription of *Cyp6g1* in embryos. These data indicate that by the late embryo stage, the maternally derived higher levels of *Cyp6g1* transcript are masked by high levels of *Cyp6g1* expression.

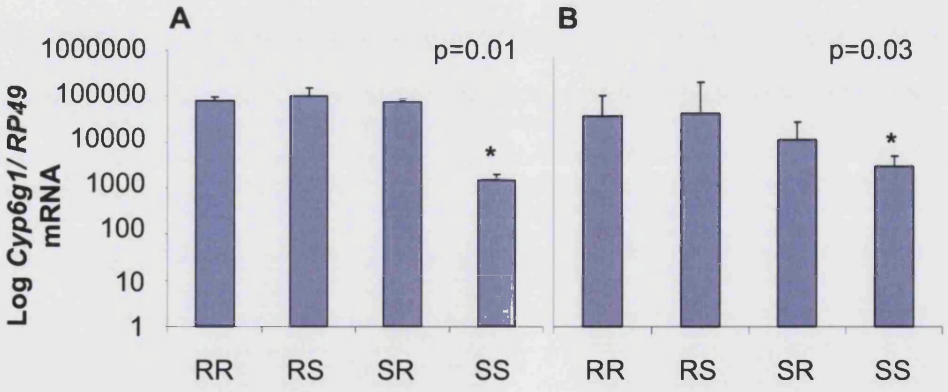
Future work that can be used to confirm the maternal transmission would be to inhibit transcription of *Cyp6g1* in embryos. The presence of *Cyp6g1* mRNA in

Figure 4.8 Quantity of *Cyp6g1* mRNA in early and late embryos



*The amount of mRNA of each strain at A. 3 hours and B. 18 hours is plotted as a ratio of Cyp6g1 to the ribosomal protein RP49. * and ** indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons) and the p value is given.*

Figure 4.9 Quantity of *Cyp6g1* mRNA in early and late larvae



*The amount of mRNA of each strain at A. 1st instar and B. 3rd instar is plotted as a ratio of Cyp6g1 to the ribosomal protein RP49. * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons) and the p value is given.*

embryos with transcriptional inhibition would indicate maternally inherited mRNAs are present. RNAi can be used to knock down the transcription of the gene. Using a GAL4/UAS based RNAi strain; the transcription of *Cyp6g1* may be knocked down in the embryo whilst the transcription of *Cyp6g1* within the maternal parent would be maintained as normal.

4.3.2.2 Quantity of mRNA in early and late stage larvae

When the amount of *Cyp6g1* transcript is quantified in 1st and 3rd instar larvae there is no significant difference between RR, RS and SR genotypes (figure 4.9), whilst the amount of transcript is significantly lower in SS flies ($F=18.4$, $p=0.01$ and $F=9.23$, $p=0.03$). The quantity of *Cyp6g1* mRNA relative to *RP49* is 100 fold higher than in late embryos indicating high levels transcription of *Cyp6g1* in the larvae. High expression in the larvae is not surprising since *Cyp6g1* over expression confers resistance to insects which act as larvicides e.g. imidacloprid (Daborn, Boundy et al. 2001). The effect of over expression appears in the larvae to be dominant, as is resistance to imidacloprid.

4.3.2.3 Quantity of mRNA in pupae and adult flies

Quantification of *Cyp6g1* transcript in pupae indicated a lower abundance of transcript compared with in the larval stages (figure 4.10). There is no significant difference between the genotypes ($F= 13.53$, $p= 0.61$). This would be explained by the high variability between the genotypes, presumably due to the changing nature of the pupa. The expression of proteases within the pupae, which enable metamorphosis, would hamper the RNA extraction. It may be that there is an increase in *Cyp6g1* expression during pupal development and since this study did not separate pupae of different developmental states, the quantity of *Cyp6g1* transcript present is a reflection of the developmental stage of the pupae.

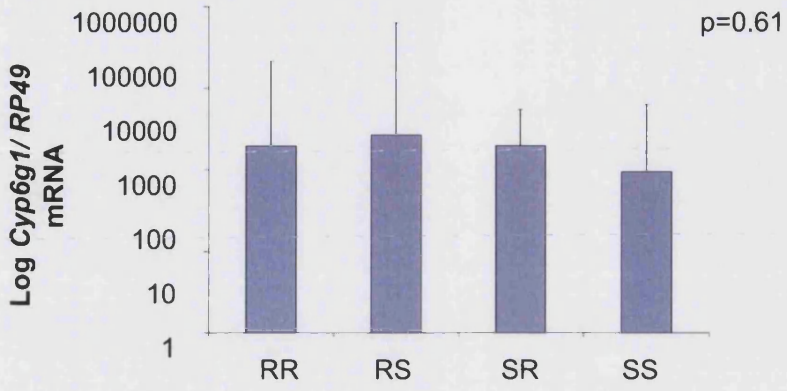
The quantity of *Cyp6g1* mRNA is significantly higher in the RR, RS and SR adult flies (figure 4.11), compared with SS ($F= 22.25$, $p< 0.01$). Consistent with the data generated in 2.3.2 the *Cyp6g1* transcript is around 100 fold more abundant

in the resistant genotypes. This result is unsurprising since the over expression of *Cyp6g1* has shown to result in resistance to DDT and resistance to DDT is a dominant trait.

4.2.4 Conclusions from analysis of *Cyp6g1* expression over *Drosophila* lifecycle

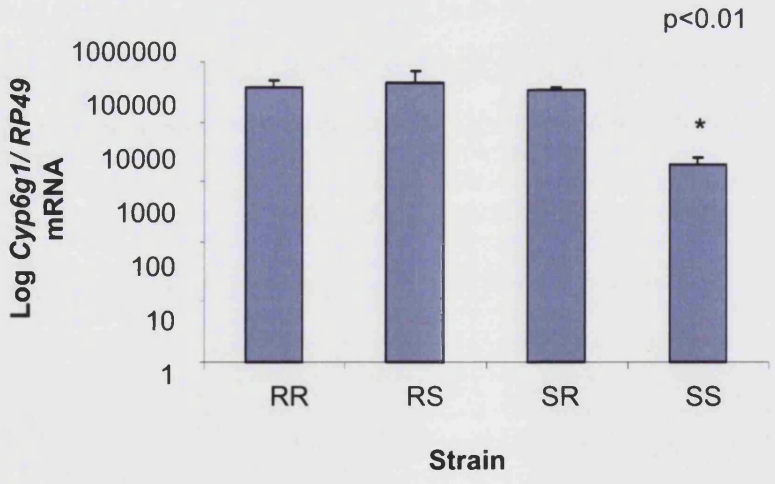
These data show that *Cyp6g1* is over-transcribed throughout the life cycle of *D. melanogaster*. The amount of *Cyp6g1* transcript is higher throughout the lifecycle of resistant insects, except at the pupal stage where high variability in transcript levels was recorded. There is a detectable level of *Cyp6g1* transcript present in all of the SS life cycle stages however this is up to 100 fold less than in the resistant genotypes. It is interesting to note that larval *Cyp6g1* expression is high in both resistant and susceptible larvae, perhaps indicating that CYP6G1 is important in the larvae. Quantification of *Cyp6g1* mRNA indicates that there may be some maternal transmission of *Cyp6g1* mRNA to embryos. This maternal transmission may be important in explaining the fitness advantage observed in embryos and larvae of *D. melanogaster* with a resistant female parent. The data do not show a difference in transcript abundance between RS (resistance allele inherited from the female parent) and SR (resistance form the male parent) in all other life history stages studied, indicating that the increased maternal transmission in resistant flies may be sufficient for the increased fitness observed in Chapter 3. The data do not eliminate the possibility that the fitness advantage seen in resistant flies is due to another closely linked trait. Follow up work is required to confirm the maternal *Cyp6g1* mRNA transmission.

Figure 4.10 Quantity of *Cyp6g1* mRNA in pupae



*The amount of mRNA of each strain is plotted as a ratio of Cyp6g1 to the ribosomal protein RP49. * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons) and the p value is given.*

Figure 4.11 Quantity of *Cyp6g1* mRNA in adults



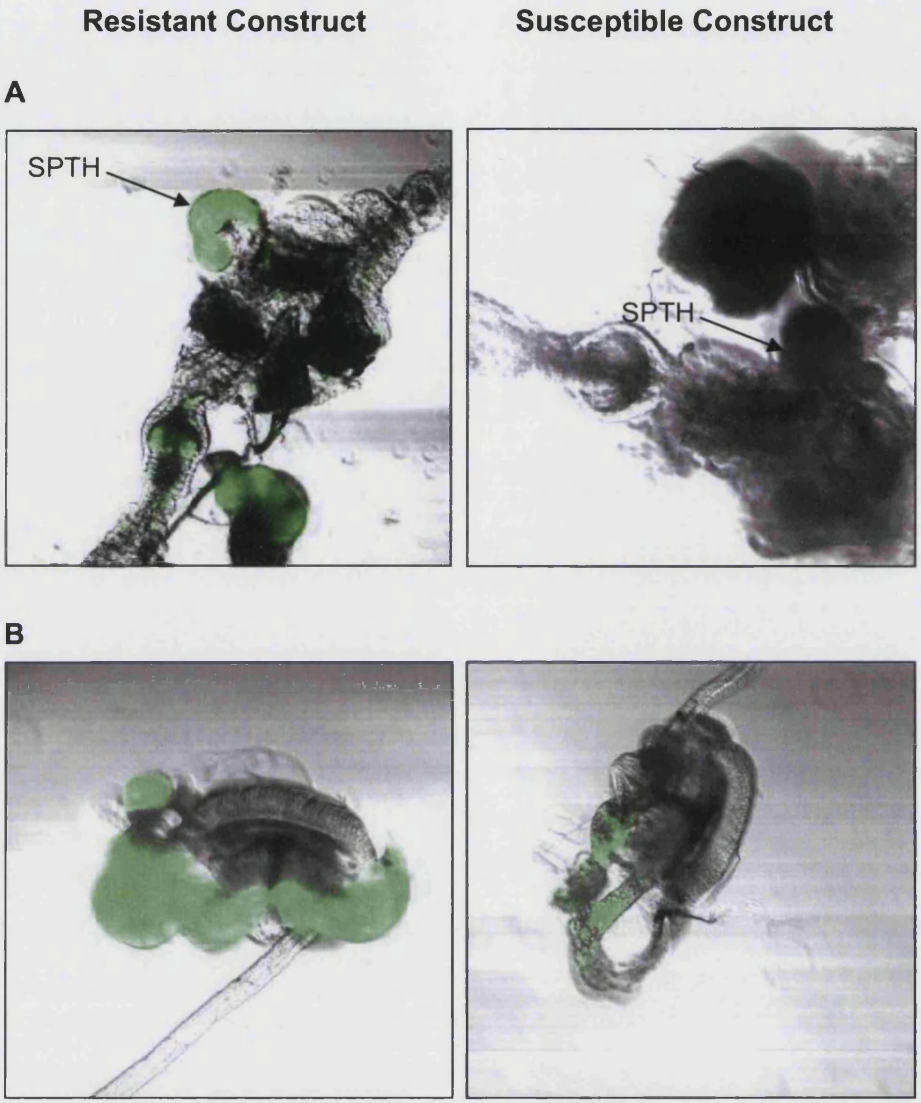
*The amount of mRNA of each strain is plotted as a ratio of Cyp6g1 to the ribosomal protein RP49. * indicates that the differences between the genotypes are significant at the 5% level (ANOVA, with Tukey post-hoc pairwise comparisons) and the p value is given.*

4.3.3 Expression of *Cyp6g1* in reproductive tissues

The GAL4/UAS expression system was used to express GFP regulated by the 5' of *Cyp6g1* allowing a comparison of expression in flies with the resistant and susceptible regulatory sequences. Initial results indicated that in female flies there is strong GFP expression in the spermathecae (figure 4.12 A). The spermatheca is an organ of the female reproductive tract in insects. Its purpose is to receive and store sperm from the male, and it is usually the site of fertilization when the oocytes are ready (Pitnick, Markow et al. 1999). The sperm storage organ allows the special separation of the sperm of different males allowing the female choice over the male that sires the offspring (Pitnick, Markow et al. 1999). Low levels of expression were observed within the ovaries of the resistant construct females. Very low levels of GFP expression was observed in the ovaries of susceptible construct females. The developing embryos within the females showed low levels of GFP expression, mainly concentrated around the posterior appendages (figure 4.13). This is consistent with the Q RT-PCR data, which also indicated low levels of *Cyp6g1* transcript within the embryo.

In the male reproductive tissues, strong GFP expression was observed in the testes and the seminal vesicles (figure 4.12 B. The seminal vesicles are the sperm storage organs in the male fly and the testes are the site of sperm production (Lefevre and Jonsson 1962). The initial dissections used mated females. When unmated females were examined, the strong GFP expression seen in the spermathecae of mated resistant females was no longer evident, indicating that the GFP expression observed in the resistant construct flies was in the sperm contained within the spermathecae and not the tissue of the spermathecae. There was no difference in the GFP expression in the female reproductive organs of resistant and susceptible virgin flies. It is however, possible that the presence of sperm in the spermathecae induces *Cyp6g1* expression in the spermatheca. To test this, virgin female flies expressing GFP under the control of the resistant and susceptible drivers were mated to males of the strains paired, which produce sperm but no accessory gland proteins and Son of Tudor, which produce accessory gland proteins but no sperm. No GFP was observed in the spermathecae of these females indicating that neither the

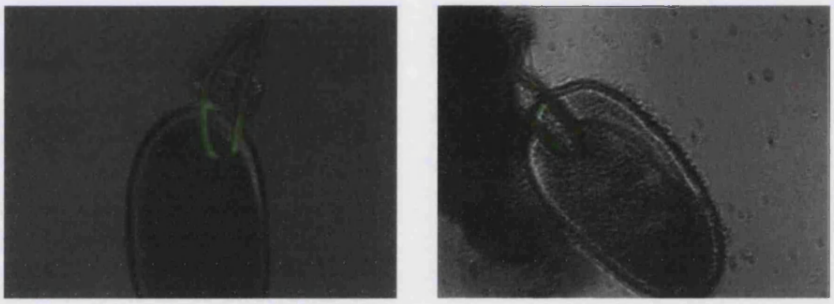
Figure 4.12 GFP expression driven by GAL4/UAS expression system using driver strains with 5' Hikone-R (resistant) and 5' Canton-S (susceptible) promoter regions.



Confocal images comparing resistant and susceptible constructs A. female reproductive tissues, the spermathecae are indicated by an arrow B. male reproductive tissues

Figure 4.13 GFP expression driven by GAL4/UAS expression system using driver strains with 5' Hikone-R (resistant) and 5' Canton-S (susceptible) promoter regions.

Resistant Construct **Susceptible Construct**



Confocal images comparing embryos of flies expressing the resistant and susceptible constructs.

presence of sperm nor accessory gland proteins in the spermatheca was inducing the expression of GFP previously seen in the spermatheca.

4.3.4 Expression of *Cyp6g1* in sperm

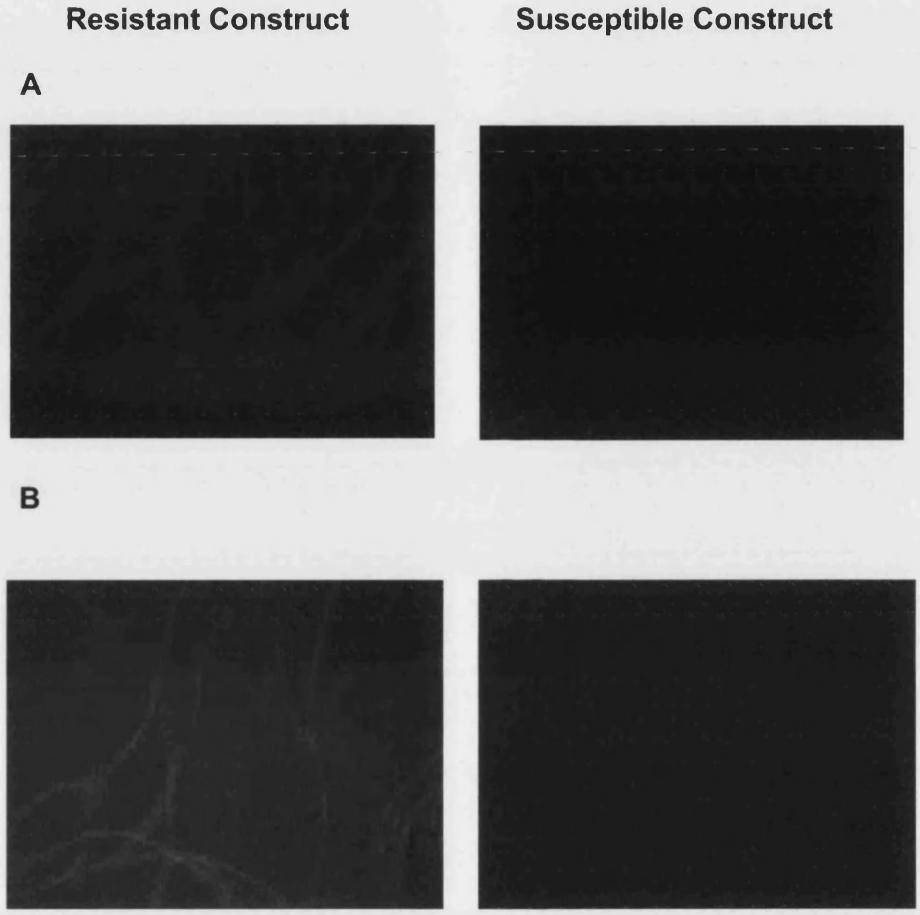
4.3.4.1 GFP expression in sperm

Sperm was extracted from the seminal vesicles of flies, washed and examined for GFP expression. The sperm from the flies expressing GFP driven by the resistant construct appear to be expressing GFP in the isolated sperm (figure 4.14 A). The sperm from the flies expressing GFP driven by the susceptible construct appear to be expressing GFP in the isolated sperm but the intensity is much lower than in the resistant construct sperm, indicating that *Cyp6g1* is expressed at a lower level in susceptible sperm compared with resistant sperm. The GFP expression appears to be in the flagella of the sperm. This is an interesting observation as the GFP is a nuclear expressing GFP. This indicates that the GFP may be translocated from the nucleus to the cytoplasm of the sperm. To confirm that GFP is being expressed in the sperm and that the fluorescence observed is not due to autofluorescence produced by the sperm, an antibody raised to GFP was applied to the sperm.

4.3.4.2 GFP antibody

To allow for natural variation between samples, a large number of samples were prepared and viewed and two images representative of preparations from the two genotypes are shown. The resistant construct samples treated with the GFP antibody showed higher fluorescence when compared with the susceptible construct sperm (figure 4.14 B). The results confirm that GFP is expressed in the resistant and susceptible construct sperm and that the expression is greater in the resistant construct sperm. The use of the UAS GAL4 expression system is useful in indicating patterns of expression however there is the possibility that the expression system is inducing expression in tissues which do not express

Figure 4.14 GFP expression driven in sperm by GAL4/UAS expression system using driver strains with 5' Hikone-R (resistant) and 5' Canton-S (susceptible) promoter regions.



Confocal images comparing sperm A. expressing GFP under the control of the resistant and susceptible constructs and B. anti-GFP antibody binging to resistant and susceptible construct sperm

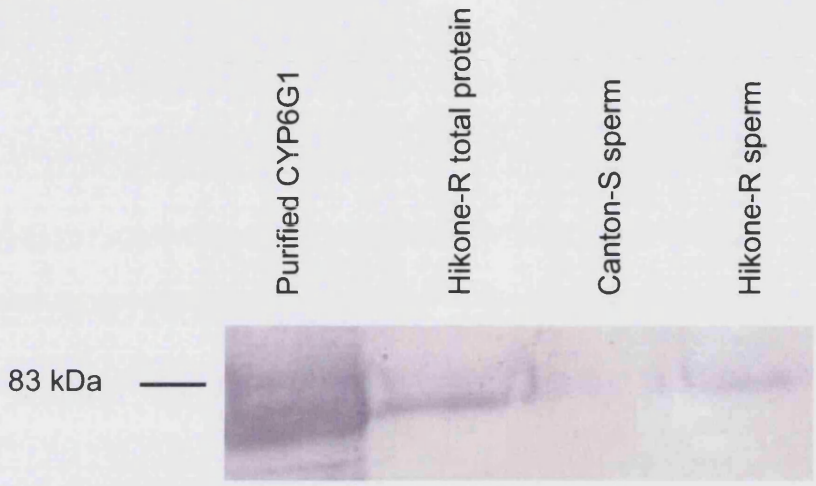
Cyp6g1. To confirm that *Cyp6g1* is expressed in sperm, western blotting was used to detect CYP6G1 in isolated sperm.

4.3.4.3 Western blotting of sperm with *Cyp6g1* antibody

The *Cyp6g1* antibody is a polyclonal antibody from rabbit serum and showed high cross reactivity to an number of proteins on the membrane. Initial attempts to perform western blots were hampered by this strong cross reactivity. Attempts to purify the antibody produced more successful blots. In addition, to reduce the non-specific binding the most successful method involved including 5% milk powder in the antibody washes to block the membrane. After these measures there was still non-specific binding but this was much reduced. Using purified CYP6G1 as a positive control allows the identification of a band of the correct size. The western blot of sperm from Hikone-R and Canton-S using the *Cyp6g1* antibody detected a band in the isolated sperm from both Canton-S and Hikone-R (figure 4.15) at the same height as the purified CYP6G1.

The data from the GFP studies and the western blot indicate that *Cyp6g1* is expressed in sperm. The role of *Cyp6g1* in sperm is not likely to be important in conferring DDT resistance since the *Cyp6g1* expression in sperm would produce only small quantities of the protein, in comparison with the larger tissues of the midgut and fat body. Over expression of *Cyp6g1* has been linked to reduced male reproductive success (Drnevich, Reedy et al. 2004). The overexpression of *Cyp6g1* in sperm is unlikely to result in the increased fecundity of the resistant females since the increased fecundity of RR females is unaffected by male genotype (figure 3.6).

Figure 4.15 Western blot of CYP6G1 using CYP6G1 polyclonal antibody



Western blot was performed with purified CYP6G1, total protein extracted from Hikone-R flies and protein extracted from isolated Hikone-R and Canton-S sperm.

4.3.5 Conclusions

This chapter aimed to address the results of the previous chapter, which indicated that the overexpression of *Cyp6g1* confers a fitness advantage to offspring with a resistant female parent. This chapter provides evidence that there is maternal transmission of *Cyp6g1* mRNA. However genetically manipulating *Cyp6g1* did not have an effect on the fecundity of flies. Preliminary work found no clear effect of juvenile hormone on the fecundity of flies, however further work is needed to confirm or reject any involvement of juvenile hormone on fecundity. It would be interesting to look at the effect of juvenile hormone on the viability and developmental rate of flies since JH has been shown to have an important role in moulting and development in the insect(Flatt, Tu et al. 2005). GFP studies indicate that *Cyp6g1* is expressed at low levels in female reproductive organs, however no difference was observed between resistant female and susceptible female constructs. The GFP studies indicated that *Cyp6g1* is expressed in sperm and this result was confirmed by western blotting. The role of *Cyp6g1* in sperm is not obvious and further work would be necessary to determine the putative function of *Cyp6g1* in reproduction.

CHAPTER 5

Resistance to dieldrin in *Ctenocephalides felis*

5.1 INTRODUCTION

5.1.1 Controlling *C. felis* populations

The annual expenditure on flea control products in the US exceeds US$1 billion and in Western Europe exceeds €1.1 billion (Rust 2005). Early control measures required the treatment of the indoor and outdoor environments as well as the infested pet. More recently systemic and topical insecticides such as lufenuron, fipronil and imidacloprid have all been used in flea control. These have the advantage of requiring treatment of the animal only, either orally or topically.

Compounds currently in use include selamectin, a monosaccharide avermectin. Avermectins do not show activity towards fleas in artificial membrane studies (Zakson-Aiken, Gregory et al. 2001) however, selamectin at a dose rate of 6 mg/kg has been shown to kill >98% of fleas within on topical application on cats (McTier, Shanks et al. 2000) and was discovered using a flea membrane-feeding assay (Banks, Bishop et al. 2000). Selamectin has been shown to provide nearly 100% protection for up to three months in flea infested environments (Ritzhaupt, Rowan et al. 2000; Ritzhaupt, Rowan et al. 2000). Selamectin is marketed as Stronghold™ in Europe and Revolution™ in the United States and has the added advantage of controlling heartworm and some gastrointestinal nematodes (Bishop, Bruce et al. 2000).

Imidacloprid (the active ingredient of Advantage™ flea treatments) is commonly used to treat pets against flea infestations. As a chloronicotinyl insecticide, imidacloprid inhibits the nicotinic acetylcholine receptor channels. Topical application has been shown to kill >95% of fleas on cats and dogs for 28-37 days (Ritzhaupt, Rowan et al. 2000; Ritzhaupt, Rowan et al. 2000; McTier, Evans et al. 2003). Sufficient amounts of imidacloprid are transferred to the immediate environment of the pet and this has also been shown to reduce the number of off-host life stages by >98% (Dryden, Perez et al. 1999).

Fipronil (the active ingredient of Frontline™), is a phenylpyrazole insecticide that has been used since the mid-1990s for controlling indoor pests (Rust 2005). Once applied to the animal the sebaceous glands in the skin serve as the

reservoir and therefore the compound does not become systemic. Frontline™ was first launched in 1984 is used to treat both dogs and cats and is now the world's best selling flea and tick treatment for those species (Merial, 2006). Fipronil acts as an antagonist by stabilizing non-conducting conformations of the GABA-gated chloride channels in the CNS as described in Chapter 1. This has the effect of blocking the GABA-gated chloride channel and reduces neuronal inhibition, which leads to hyperexcitation of the central nervous system, convulsions, and death. The potency of fipronil is dependent on the GABA concentration but is unaffected by membrane potential (Hosie, Baylis et al. 1995).

5.1.2 *Resistance to dieldrin*

The *Resistance to dieldrin (Rdl)* gene encodes a subunit of the insect GABA receptor (ffrench-Constant, Rocheleau et al. 1993). The RDL subunit assembles with other GABA receptor subunits to form the target site of the cyclodiene and phenylpyrazole insecticides (ffrench-Constant, Anthony et al. 2000). Target site resistance is associated with point mutations in *Rdl* that predict replacement of alanine302 with either a serine or a glycine (ffrench-Constant, Anthony et al. 2000) as described in more detail in Chapter 1 (figure 1.3). The mutation replacing alanine302 is highly conserved throughout a range of different insect orders (ffrench-Constant, Anthony et al. 2000). This includes a number of pest species such as the German cockroach, *Blattella germanica,* the mosquito *Anopheles gambiae* the American cockroach, *Periplaneta americana* (L), and the peach potato aphid, *Myzus persicae* (ffrench-Constant, Anthony et al. 2000). This mutation was originally associated with cyclodiene resistance however electrophysiological studies on RDL receptors expressed in Xenopus oocytes, (Hosie, Baylis et al. 1995) have shown that the A301S mutation, strongly reduced the potency of fipronil as well as dieldrin and picrotoxin (PTX) (ffrench-Constant et al. 1993a).

5.1.3 Resistance diagnostics

Due to the widespread conservation of *Rdl* resistance-associated mutations, numerous different techniques have been used to detect resistance-associated point mutations. These are useful in monitoring the spread and prevalence of resistance in a population. First, PCR followed by a diagnostic Restriction Endonuclease analysis (PCR REN) may be used in determining the frequency of the resistant allele. The single-base-pair substitutions in exon 7 both result in the creation of a *BsmAI* restriction enzyme site in the resistant allele. This restriction enzyme site serves as a marker of the resistance phenotype. When a PCR product encompassing exon 7 is digested with *BsmAI, SS* strains should yield one product but *RR* strains yield two products and *RS* yields all three (Daborn, McCart et al. 2004). In *D. melanogaster* the resistance associated point mutation deletes a *HaeII* resetricition site.

Polymerase chain reaction (PCR) based Amplification of Specific Alleles (PASA) is capable of genotyping individual insects and identifying specific resistance alleles (Steichen and ffrench-Constant 1994). The method requires the resistance mutation to be placed at the 3' end of the allele specific oligonucleotides. Other alleles will not amplify well due to the mismatching of the primers and the template and therefore allows the identification of the detection of the resistance-associated mutation. The technique does not require the presence of a resistance associated restriction enzyme polymorphism. In addition PASA allows the determination of the frequency of different alleles, unlike PCR REN, which only establishes the overall resistance gene frequency. PASA cannot be used for resistance alleles with unknown nucleotide sequence, however and more than one reaction is required to obtain a genotype for and individual.

Single Stranded Conformational Polymorphism (SSCP) analysis is a technique that is capable of detecting all mutations on a stretch of DNA. The technique is a sensitive but inexpensive and rapid, method for determining which DNA samples in a set differ in sequence, so that only a subset need be sequenced (Sunnucks, Wilson et al. 2000). The technique exploits the differing secondary structure of single-stranded nucleic acids based on subtle differences in sequence and the

resulting measurable difference in mobility through a gel of the differing conformations. The technique has the advantage that it can be used to detect novel resistance mutations in genes of unknown sequence (ffrench-Constant, Aronstein et al. 1995) and has been successfully applied to the detection of the *Rdl* alleles in *D. melanogaster and D. simulans* (Coustau and ffrench-Constant 1995).

The disadvantage of all of these techniques is that none of them are high throughput or readily automated. In this chapter I will describe the use of a TaqMan 5' nuclease assay to detect resistance-associated mutations (Daborn, McCart et al. 2004). This method is unique due to its high-throughput potential. The technique allows allelic discrimination of an insecticide resistance-associated mutation in cat flea (*C. felis*) Rdl using fluorogenic probes and a TaqMan assay. In this assay a fluorogenic probe, consisting of an oligonucleotide labelled with both a fluorescent reporter dye and a quencher dye, is included in a typical PCR (Livak 1999; McGuigan and Ralston 2002). Amplification of the probe-specific product causes cleavage of the probe, generating an increase in reporter fluorescence. By using different reporter dyes for susceptible (S) and resistant (R) alleles, cleavage of allele-specific probes can be detected in a single PCR (figure 5.2). All TaqMan genotyping assays can be performed using a single reaction buffer and thermocycling protocol, facilitating high-throughput analysis and automation (Shi, Bleavins et al. 1999; Hiratsuka, Agatsuma et al. 2000).

5.1.4 Aims of this chapter

The aims of this chapter are to develop a TaqMan based assay to detect the resistance allele in single fleas. The TaqMan assay will be applied to fleas sampled from the field and laboratory to test for resistance within these populations. This will provide information on the prevalence and distribution of the resistant allele, which is particularly useful for resistance management. In addition the aim it to try to establish a correlation between the resistance allele and resistance to cyclodiene insecticides, in particular dieldrin and the phenylpyrazole fipronil.

5.2 MATERIALS AND METHODS

5.2.1 Flea strains used

Fleas were collected from field sites in Kent during July and August 2003. Untreated cats were defined as having no insecticidal treatments in the six months prior to sampling. Field collected fleas were taken directly from infested cat hosts. Fleas were sampled from laboratory strains established in the USA and UK. The source, location and treatment status of the strains used is shown in table 5.1.

Table 5.1 Source and treatment status of fleas

Strain	Source	Treatment
Laboratory[a]	North Carolina	None
Laboratory[a]	California	None
Laboratory[a]	Germany	None
Laboratory	Ireland	None
Laboratory	England	None
Field collection	Kent	3 untreated cats[b]
Field collection	Kent	4 treated cats[c]
Field collection	California	6 cats[d]
Field collection	California	10 dogs[d]

[a] Denotes laboratory strain supplemented with field material
[b]Untreated cats were from Broadstairs, Ash and Eastry, Kent, UK
[c]Treated cats were from Kingsdown (two) and Canterbury Kent, UK and were treated with fipronil ('Frontline').
[d]Treatment status unknown

5.2.2 Cloning and sequencing of *Rdl*

Phillip Daborn performed the cloning and sequencing of *Rdl* to allow for the development of the TaqMan diagnostic. For DNA extraction, single fleas were homogenized in 50µl of 10 mM Tris pH 8.2, 25 mM NaCl, 1 mM EDTA, 200µg/ml proteinase K with a polypropylene pellet pestle (Sigma) in a 1.5 ml Eppendorf tube. Homogenates were then incubated at 37°C for 30 min, and 85°C for 10 min. 1 µl was used for PCR. Exon 7 of *Rdl* was amplified using the primers Ex7F (GAA ACT ATT CTC GCC TGG CGT GCG AG) and Ex7R (CTA ATA AAC TAG CGA ACA CCA TTA CG), with an annealing temperature of 57°C for 1 min,

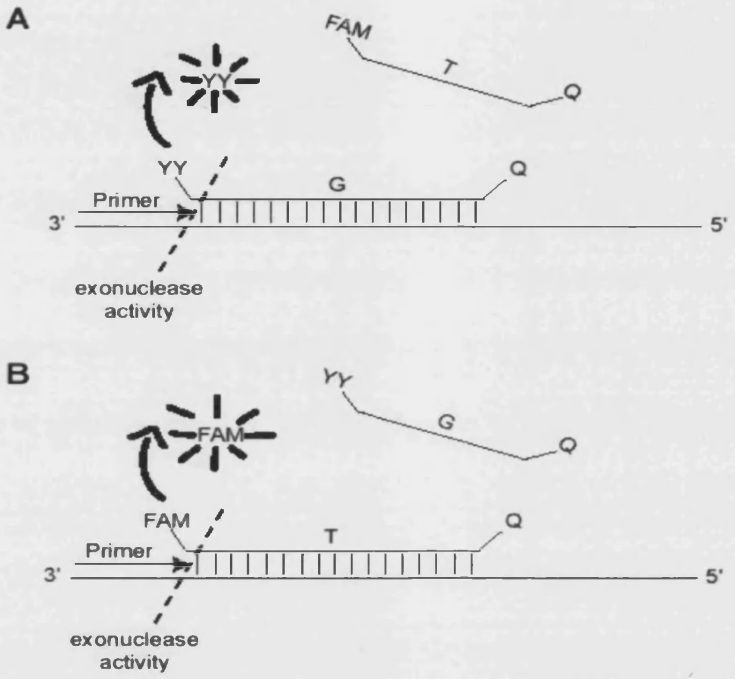
and an extension time of 1 min at 68°C, using Taq DNA Polymerase (Applied Biosystems) following manufacturers instructions. Resulting PCR products were cloned into pUC18 digested with SmaI. Both DNA strands of clones were then sequenced. Following the identification of a point mutation predicting the replacement of alanine302 with a serine it was possible to design the PCR based diagnostics.

5.2.3 TaqMan diagnostic

The TaqMan assay was used for genotyping individual cat fleas. In this assay two different reporter dyes (FAM for resistant and Yakima Yellow for susceptible) are attached to allele specific probes. A quencher is attached to the 3' end of the probe. The probe and primer sequences were designed with Beacon Designer 2.1 (Premier Biosoft International, Palo Alto, Calif.). The primers (SNP-F 5'-ATC GTT TTG GCT GAA TCG TAA TG-3' and SNP-R 5'-GAC ATT AGA GTT GTC ATG GTC ACC-3'), (MWG, UK) amplify a 66 bp product encompassing the resistance-associated mutation. The fluorescent probes 5'-FAM-ACCAGCTCGAGTCTCTCTCGGAGT-TAMRA-3' and 5'-YakimaYellow-ACCAGCTCGAGTCGCTCTCGGAGT-Eclipse-3' (Eurogentec, UK) were used for the resistant and susceptible genotypes, respectively (figure 5.1). The resistance-associated mutation is underlined. Amplification of the probe specific product causes cleavage of the probe and separation of the corresponding allele specific dye from the quencher. Individuals can then be genotyped according to the fluorescence from one dye or the other (*RR* or *SS*), or the fluorescence of both dyes (*RS*).

For DNA extraction, single fleas were homogenized in 50µl of 10 mM Tris pH 8.2, 25 mM NaCl, 1 mM EDTA, 200µg/ml proteinase K in a 96 well plate (Fisher, UK) with a customised 96 well homogeniser (Fisher, UK). Homogenates were then incubated at 37°C for 30 min, and 85°C for 10 min to denature the proteinase. PCR mixtures of 20 µl, containing 1 µl of DNA from single fleas, were used for hybridisation probe PCR using the QuantiTect Probe PCR Kit (Qiagen), and a range of fluorophore concentrations. A fluorophore concentration of PCR was performed and fluorescence detected using a DNA Engine Opticon 2 System (MJ

Figure 5.1 Design of the TaqMan diagnostic for flea *Rdl*.



Allele specific probes are designed for both the S and R alleles, each carrying a different fluorescent dye (FAM for resistant and Yakima Yellow for susceptible) and quencher. After PCR amplification the specific products are cut by the 5' nuclease and their fluorescent dyes released. The amount and presence of the dye indicates the genotype.

Research). The PCR conditions were as follows: 95°C for 15 min to activate the hot-start DNA polymerase, followed by 40 cycles of 95°C for 15 s for denaturation and 66°C for 1 min combined annealing and extension. Fluorescence was monitored each cycle. During the extension phase of PCR, if the probe binds to the target sequence, it is cleaved by the 5'→3' exonuclease activity of the DNA polymerase, separating the fluorophore from the quencher at the 3' end. This leads to fluorescence being emitted. Fluorescence was monitored each cycle and recorded by the Opticon monitor software.

5.2.4 Bioassay of fleas

A population of *Ctenocephalides felis* from Germany was found to have an *Rdl* frequency of approximately 60% RR and was identified as a suitable strain for bioassays due to the higher frequency of the *Rdl* resistant allele. The German laboratory strain was cultured in vivo. To allow the fleas to be cultured for selection and bioassay experiment the strain was adapted to membrane feeding at Pfizer. This strain was compared with the laboratory strain already in cultured in vitro at Pfizer. The RDL RR frequency of this strain was found to be <3%. Fipronil and dieldrin were prepared at 0.3, 0.1, 0.03, 0.01, 0.003, 0.001 µg/ml in acetone. 200 µl of the insecticide was pipetted into the vials and the vials rolled until the acetone had evaporated. Controls with 200 µl acetone only were also rolled. Five vials per repeat and five repeats were set up. 30 fleas per vial were added and the lids loosely screwed on. Vials were left for 24 hours at room temperature before the number of dead fleas were scored. Fleas lying on their backs twitching were counted as dead.

Fleas that survived the bioassay were separated from the non-survivors. All bioassay fleas were snap frozen in liquid nitrogen and stored at -80 °C before the DNA extraction and TaqMan assay were performed as described previously.

5.3 RESULTS AND DISCUSSION

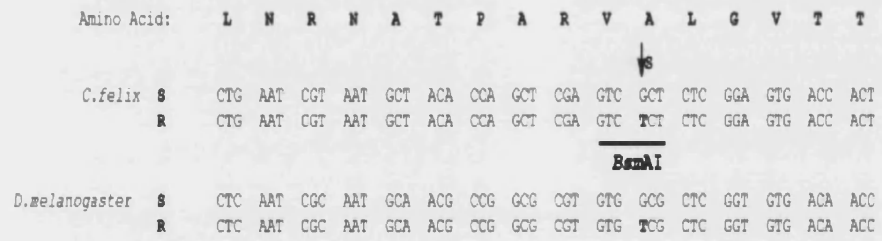
5.3.1 Cloning and sequencing of *Rdl*

Exon 7 of cat flea *Rdl* was cloned and sequenced by Phillip Daborn. As predicted this region of amino acid sequence, which is believed to line the integral chloride ion channel of the GABA receptor, is highly conserved with other insects (ffrench-Constant, Anthony et al. 2000). In common with insects from several other orders (ffrench-Constant, Anthony et al. 2000), field populations of the cat flea also show putatively resistance-associated point mutations with *Rdl* (figure 5.2). These mutations all predict the replacement of alanine302 with a serine. No other predicted replacements, such as glycine, were detected in the limited sample numbers. This concurs with more recent work where the A302 to S302 was the only base difference observed in this region following repetitive sequencing of multiple individuals from several strains (Bass, Schroeder et al. 2004). The alanine302 serine replacement causes resistance in functionally expressed RDL homomultimers cloned from a variety of insects (ffrench-Constant, Rocheleau et al. 1993), and also correlates well with resistance in several insect field populations (Aronstein, Ode et al. 1994). The successful cloning and sequencing of *Rdl* allows for the development of the TaqMan based diagnostic.

5.3.2 TaqMan diagnostic

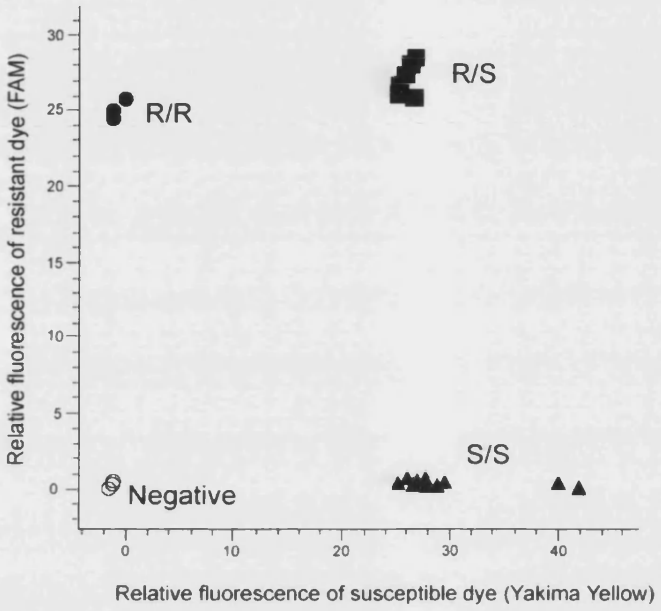
The TaqMan based assay gives good discrimination between *SS, RS* and *RR* individuals (figure 5.3). The results appear to be unambiguous in all cases. Cloned and sequenced DNA was used as positive and negative controls. The assay consistently gave the correct result for these controls over the large number of repeats that were used, despite only a single amino acid difference. A far greater number of sequenced samples would have to be run to determine a failure frequency, however this would be costly and since the assay has been accurate (either giving the correct or no result) for over 200 repeats the results can be considered to be very accurate.

Figure 5.2 The sequence of exon 7 of a susceptible (*S*) and resistant (*R*) allele of the *Rdl* gene from the cat flea.



Note that the resistant allele predicts the replacement of alanine302 with a serine.

Figure 5.3. Allelic discrimination of cat flea *Rdl* alleles using a TaqMan assay.



The presence of only the Yakima Yellow (susceptible specific) dye after amplification denotes an SS genotype, whereas only FAM (resistant specific) denotes RR. Detection of both dyes indicates a RS heterozygote. A water only control (negative) has been included.

The TaqMan based assay has the advantage over REN PCR that no gel needs to be run and that sample preparation and analysis can be automated to look at large numbers of field-derived insects. The assay can be scaled to run 96 or 384 samples at a time and the relatively short reaction time allows the analysis of a large number of samples relatively quickly. TaqMan based assays for the detection of resistance-associated point mutations therefore have advantages over all of the other previously developed techniques such as PCR REN, PASA and SSCP (Steichen and ffrench-Constant 1994; Coustau and ffrench-Constant 1995) all of which require analysis of PCR derived products by gel electrophoresis. The central disadvantages of TaqMan based assays for non-biomedically based analyses, are their relatively high cost and the fact that, unlike SSCP analysis, only alleles of known sequence can be detected.

The TaqMan assay is a very useful tool in the quantification of gene expression, detection of polymorphisms and quantification of DNA deletions. The quantification of very small amounts of genetic material is useful in a number of fields, in particular those in where sample is limited in abundance (for example in forensics), and when nucleic acids are at low but significant levels (Watson and Li 2005). The use of TaqMan based diagnostics for individual insects can determine the relative frequencies and spatial distributions of resistance mechanisms conferred by single base changes. This can be used to advise farmers and growers on the most effective choice of insecticide. As the costs of developing and running DNA-based diagnostics fall, and their reliability and the levels of automation improve, it is increasingly likely that PCR based tools will become an important and widely used technique in resistance detection and management in the future.

5.3.3 Frequency of *Rdl* alleles in laboratory and field populations

The TaqMan based assay was subsequently applied to a number of laboratory and field derived strains to determine the frequency of the resistant allele within a number of populations. The two laboratory strains from North America are nearing fixation for the resistant allele. The strain of fleas is the German strain, which shows a resistant allele frequency of approximately 80%. These strains

were both supplemented with fleas from the field. The Irish and English laboratory strains, which are not supplemented with fleas from the field, have a frequency of <3% for the resistant allele.

The field fleas collected from the UK are from only a very limited number of animals, however susceptible (SS) fleas were only recovered from untreated cats and all treated cats carried only *RS* or *RR* fleas (table 5.2). Given that resistance mediated by *Rdl* is dominant (ffrench-Constant 1994), both these genotypes could therefore have potentially survived treatment with fipronil. The fleas collected from animals in California were from a fairly small sample of animals from the areas of Modesto, Turlock, Lake Forest, Ceres, Hughson and Mission Viego all located approximately 50 miles east of San Francisco. The treatment status of the animals that the fleas were collected on is unknown. The results show all but two of the 175 fleas collected were homozygous for the putative resistant allele indicating that the resistant allele is reaching fixation in this particular area. These results suggest that the use of field fleas to supplement the lab population is maintaining the resistant allele within laboratory populations.

The two susceptible individual fleas represent a SS allele frequency of 1.1%. Since the resistance mediated by *Rdl* is known to be dominant it is interesting to note that the susceptible allele is found in the population despite no heterozygous individuals. According to Hardy-Weinberg principles and with *Rdl* mediated resistance being dominant it would be expected there would be a larger number of heterozygotes, which would maintain the SS genotype within these populations. However one of the assumptions of the Hardy-Weinberg equilibrium is that there is no selection and within the field populations of fleas it can be assumed that there is selection through the use of insecticides. The small number of putative susceptible fleas may have been collected from isolated populations, although this would seem unlikely. The TaqMan assay gives a clear indication of genotype so interpretation of the results is unlikely to be a problem. There were a total of 89 out of a total of 264 fleas that did not give a result. This is probably due to poor storage of the fleas after collection resulting in degradation of the DNA and therefore the amplification and detection of the allele was not possible. The fleas that did not give a result would not be expected to consist of any one genotype more than the others and I would not expect the

PCR failures to influence the results in any way other than reducing the sample size.

Cyclodiene insecticides have been largely withdrawn from use as commercial insecticides and were not used widely for the direct control of fleas (Bass, Schroeder et al. 2004). These compounds have, however been used in various home (eg cockroach, ant and termite) and garden insect control products, and this, combined with their environmental persistence, makes it likely that these fleas would have undergone indirect exposure to cyclodienes in the past, providing a strong selection pressure for the resistant allele.

Table 5.2. Genotype of fleas all fleas collected, determined by the TaqMan based diagnostic

Strain result	Source	Treatment	RR	RS	SS	No
Laboratory[a]	North Carolina	None	8	2	0	0
Laboratory[a]	California	None	75	2	0	0
Laboratory[a] (pupae)	Germany	None	0	0	0	6
Laboratory[a] (dead)	Germany	None	104	26	1	51
Laboratory[a] (alive)	Germany	None	63	27	15	75
Overall: Laboratory[a]	Germany	None	167	53	16	132
Laboratory	Ireland	None	2	1	120	19
Laboratory	England	None	2	3	168	92
Field	Kent	3 untreated cats[b]	9	3	3	1
Field	Kent	4 treated cats[c]	4	3	0	3
Field	California	6 cats[d]	110	0	1	24
Field	California	10 dogs[d]	63	0	1	65

[a] Denotes laboratory strain supplemented with field material
[b]Untreated cats were from Broadstairs, Ash and Eastry, Kent, UK
[c]Treated cats were from Kingsdown (two) and Canterbury Kent, UK and were treated with fipronil ('Frontline').
[d]Treatment status unknown

The German strain of fleas was chosen for further work on *Rdl* as it shows a high frequency of the putative resistant *Rdl* allele and was considered suitable for selection experiments. The strain was adapted for membrane feeding at Pfizer, allowing the strain allowing the strain to be used in bioassay and selection

experiments. The high number of PCR failures was determined to be due to poor handling and storage, and when fleas were shipped live the number of PCR failures was reduced.

5.3.4 Membrane adaptation

Bioassays were performed with two strains of membrane adapted laboratory fleas. The German strain mentioned above was compared with the UK laboratory strain (table 5.2). The genotypes of the fleas used in the bioassays were determined after the experiment using the TaqMan assay. The results of this are shown in table 5.3. The frequency of the putative resistant allele is lower than the frequency in the original population of fleas. This may be due to the decreased survival of the resistant individuals when cultured in-vivo. As described in Chapter 3, resistance has often been shown to be costly in the absence of selection and although this may not always be the case, the drop in frequency of the resistance allele in this population indicated that there might be a cost to *Rdl* resistance in fleas. In contrast the mutation has been shown to persist in laboratory culture, without selection (Bass, Schroeder et al. 2004), suggests that it either has a low fitness cost in cat fleas, or that any such cost is not realised under their laboratory conditions. In other insects, an apparent absence of fitness costs has led to persistence of the *Rdl* mutation in both laboratory and field strains (Aronstein, Ode et al. 1995). There was however a large number of fleas that did not give a result with the TaqMan assay. This is much more pronounced in the German strain than the English strain. This is possibly due to the storage of the fleas although the two strains were treated and stored identically.

Table 5.3 Genotype of fleas used for bioassays

Strain	Source	RR	RS	SS	No result
Laboratory	Germany	33	19	156	105
Laboratory	England	11	10	247	46

5.3.5 Bioassays

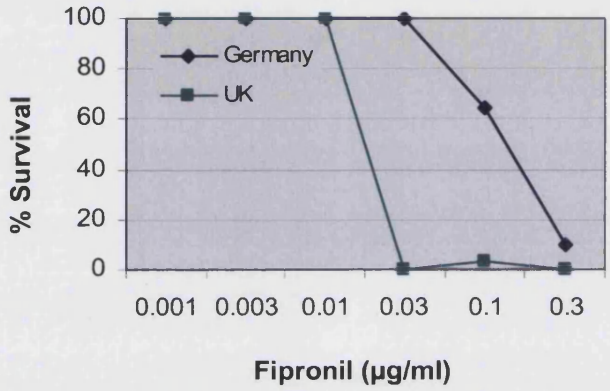
The German flea strain showed greater survival when exposed to fipronil in the bioassays when compared to the UK strain. At a dose of 0.03 µg/ml the UK strain shows 0% survival whereas the German strain shows 100% survival (figure 5.4). The dosage mortality graphs show a small shift in the response of the German strain in relation to the UK strain indicating a higher tolerance of fipronil in the German strain (figure 5.5). The results for dieldrin are similar to those for fipronil. The German strain does show an increased survival at equivalent doses of dieldrin when compared with the UK strain (figure 5.4), however the dose response curves show such a more marked shift than that seen for fipronil (figure 5.5).

The differences between the responses of the two strains are not as striking as would be expected for resistance conferred by a target site alteration. *Rdl* resistant *Blattella germanica* show 1,270-2,030 fold increased resistance to dieldrin and 15 fold increased resistance to fipronil (Kristensen, Hansen et al. 2005). The high sensitivity of the German strain to fipronil and dieldrin may be as expected since the fleas tested from the German strain have an R allele frequency of only 26% although this is higher than the UK strain, which has an R allele frequency of 6%. The two strains cannot be directly compared, as the genetic background is likely to be very different possibly affecting mortality. The UK strain is more established on the artificial dog and may therefore be healthier although the analysis includes the control mortality.

The German strain was to be pressurised with fipronil to select for the resistant allele in order to confirm that the A302 to S302 mutation confers fipronil resistance. After each stage of selection the percentage of resistant fleas could be determined using the TaqMan assay. This was not possible unfortunately as the culture was lost due to the difficulties of adapting flea strains to an artificial feeding environment. An alternative would be to create a homozygous resistant line using single pair crosses and the TaqMan assay to determine the genotype of the parents, using the offspring of only resistant parents. This is a well established technique in *Drosophila* however would be virtually impossible to achieve with fleas since there is a high level of mortality in each generation.

Figure 5.4. Survival of German and UK strains of fleas at a range of doses of fipronil and dieldrin

A



B

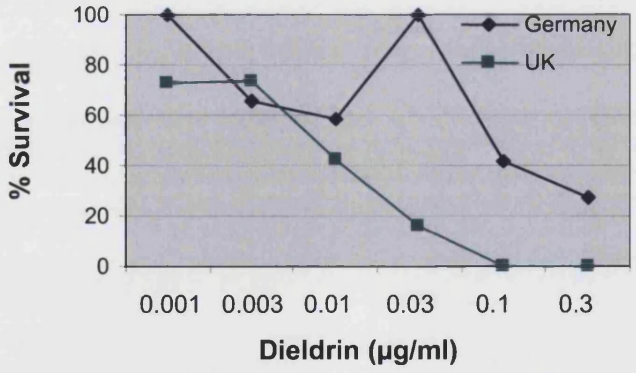
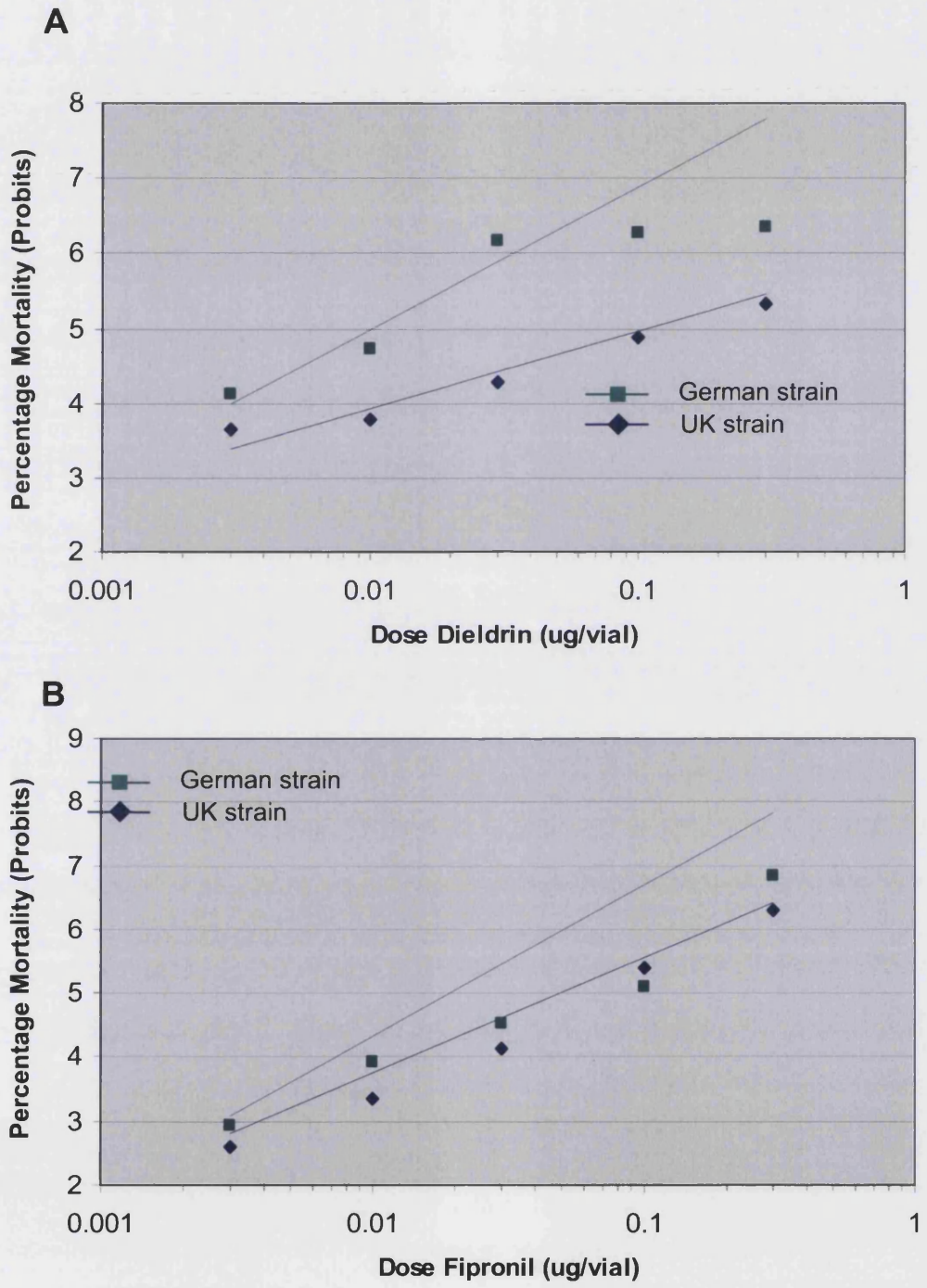


Figure 5.5. Dosage mortality curves for A. dieldrin and B fipronil tested on the membrane adapted German flea strain (shown in brown) and the UK strain.



5.3.6 Conclusions

In conclusion UK cat flea populations carry putative resistance associated mutations within *Rdl*, however, further work is necessary to confirm the correlation between the presence of these mutations and any resistance to fipronil. Such work could include several approaches. First, the use of pressurisation to select for fipronil resistance within a population of fleas with an initially low resistant allele frequency. Second, more extensive sampling of treated and untreated cats to look for evidence of control failures in the presence of a high frequency of resistance-associated point mutations. TaqMan based assays show great potential for the detection of resistance to conventional insecticides in both animal health and agriculture. The technique may also be applicable in the detection of resistance to novel insecticides such as Bt deployed in transgenic crops.

CHAPTER 6

Discussion

6.1 CONCLUSIONS FROM THESIS

The large-scale use of insecticides has enabled the increase in agricultural production for an increasing human population, the control of household pests, and the management of disease carrying vectors. Resistance is an evolutionary response to selection by toxicants resulting in impaired control in the field (Zlotkin, 1999). The complete elimination of a pest species is unlikely and therefore resistance research aims not at finding a solution to the resistance problem, but at prolonging the effectiveness of insecticides by greater understanding of the factors leading to resistance.

D. melanogaster is not a pest species, but has become an indispensable tool for the examination of almost every aspect of biology, providing a model even for distantly related species. In this thesis, the powerful genetic tools of *D. melanogaster* are used to investigate many aspects of insecticide resistance. The use of the genetic model *Drosophila* in this study allowed the use of techniques that are not available in pest species, for example the UAS GAL4 expression system. This technique allows the targeted over expression and knockdown of the gene of interest. The results further indicated a role of *Cyp6g1* in DDT resistance. The use of this expression system has enabled us to document the native expression pattern of *Cyp6g1* in adult flies using UAS GAL4 expression system and to compare the resistant and susceptible patterns of expression. The results indicate that the important tissues in DDT resistance are the midgut, Malpighian tubules and the fat body.

Using *Drosophila* has facilitated the generation of nearly isogenic fly lines differing only by the presence or absence of the resistance gene. This is an important step in the study of the fitness costs of resistance since the effects of the background genome are often neglected in fitness studies. Using *Drosophila*, rather than pest insects that are hard to cross in the laboratory, has therefore allowed us to better assess exactly when and in which genotype the cost of resistance can be measured. The results indicated that resistance does not carry a cost, and indeed may carry a fitness advantage. A more in depth look at the role of the resistance protein in reproduction indicates there may be maternal

transmission of *Cyp6g1* mRNA and that *Cyp6g1* is expressed in sperm. The role and consequences of *Cyp6g1* expression in sperm are as yet not understood.

There are few examples of thorough studies looking at the fitness costs associated with insecticide resistance. Modelling predicts resistance to be costly but makes assumptions, which in the case of *DDT-R* in *Drosophila* are not accurate. Resistance genes are assumed not to approach fixation in natural populations and the observed frequency is assumed to be the net result of the selective advantage in the presence of selection and its cost in the absence of selection. *DDT-R* in *Drosophila* however is a single mutation, which has spread globally and is now found to be fixed in populations outside of East Africa.

Although the precise molecular mechanism whereby over-expression of the CYP6G1 enzyme confers a maternally derived advantage remains to be elucidated, these results are highly significant for the population genetics of insecticide resistance. First they demonstrate that, contrary to conventional population genetic theory, insecticide resistance may show a benefit in the absence of selection, rather than a cost. This, together with the continued use of other insecticides to which *DDT-R* confers cross-resistance may help explain the fact that globally *DDT-R* is approaching fixation in non-African *D. melanogaster* populations, long after the withdrawal of widespread DDT use. These findings suggest that a re-examination of our models for managing resistance to drugs and pesticides may be necessary. Many pesticide resistance strategies rely on a reduction in the frequency of the resistant phenotype due to the assumed fitness cost associated with resistance in the absence of the pesticide. This approach may be flawed in the case of some resistance alleles as our data indicate that not all resistance alleles are costly. The implications for pesticide management are that strategies that do not assume a pesticide cost should be developed. Beyond the study of insecticide resistance, these results suggest that other xenobiotic resistance mechanisms, or indeed other adaptive traits, may not necessarily incur significant fitness costs in the absence of the selective agent.

One of the reasons that field derived strains of *D. melanogaster* are used as a model species in many insecticide resistance studies is that they share the environment with pest species. The similarity between insect genomes would

suggest that *D. melanogaster* and pest species would have similar capacities to respond to the same insecticide selection pressures. There are a number of examples of this for target site resistances e.g. *Rdl* (ffrench-Constant, Steichen et al. 1994) where different species show similar or the same mutations in the orthologous genes. The use of model insects is invaluable in predicting resistance mutations and developing a greater understanding of the molecular basis of insecticides. However once resistance has occurred in a pest species in the field, the monitoring of resistance is important. In this study the identification of the Rdl resistance mutation, which confers resistance to cyclodienes and the compound fipronil, in the cat flea, *C. felis*, indicates control failures in the field may be due to the presence of the putative resistance mutation. In order to monitor the spread of resistance a molecular diagnostic was developed. Previous *Rdl* diagnostics include PCR REN, PASA and SSCP (ffrench-Constant, Steichen et al. 1994; Coustau and ffrench-Constant 1995), all of which require analysis of PCR derived products by gel electrophoresis.

The study shows that UK cat flea populations carry putative resistance associated mutations within *Rdl*. These results are important, as currently fipronil is one of the most widely used compounds for controlling flea populations on domestic animals (Rust 2005). The TaqMan based diagnostic is becoming increasingly popular in the detection of resistance both to conventional insecticides in animal health and agriculture and also to novel insecticides such as Bt deployed in transgenic crops (Anstead, Williamson et al. 2004). As the costs of developing and running DNA-based diagnostics dropss and the capability for automation improves it is likely that molecular diagnostics will be increasingly important in resistance detection and management. Target-site mechanisms such as *Rdl*, where resistance is caused by a point mutation in a complex membrane proteins are particularly suited for molecular diagnostics however DNA based mutation detection assays can be run alongside existing biochemical assays for metabolic resistance to enable a more informed choice of which compounds to use in control.

6.2 FUTURE WORK

A recent paper presents new data confirming the increased expression of *Cyp6g1*, which leads to insecticide resistance, is due to the presence of the *Accord* LTR insertion upstream of *Cyp6g1*. Using transgenic constructs, it can be shown that all cis-regulatory elements driving the expression of this gene are located within 1.2kb of the promoter. The work also demonstrates that the Accord LTR does not change the transcription start of *Cyp6g1* but instead carries tissue specific enhancers (Chung, Bogwitz et al. In Press). There are still unanswered questions relating to the role of the *Accord* insertion. When did the insertion arise, did it predate DDT use and was the selective pressure of DDT use responsible for the insertion?

Further work is required to prove that CYP6G1 metabolises DDT and other compounds. Work presented in Chapter 2 attempted to show this however, was unable to conclusively show that CYP6G1 alone is capable of imidacloprid metabolism. Work to investigate the metabolites of the reaction is important in understanding how one enzyme can confer resistance to many chemicals. Understanding of the interactions between CYP6G1 and the insecticide are important as *Cyp6g1* may confer resistance to insecticides that have yet to be discovered or developed. The understanding of the metabolism of compounds by this enzyme is therefore of great importance in the development of new compounds.

The native role of CYP6G1 still remains unclear. The enzyme may have evolved due to the broad range of substrates, which it can metabolise, and therefore be important in protection from plant xenobiotics or other exogenous compounds. The expression of the enzyme in midgut, Malpighian tubules and fat body under a susceptible, ancestral pattern of expression, supports this as these tissues are involved in the metabolism and detoxification of toxic compounds. However the results from Chapters 3 and 4 indicate that CYP6G1 may be important in reproduction. There are a number of Cytochrome P450s with a role in reproduction, in particular the synthesis and degradation of hormones, important in reproduction. The presence of CYP6G1 in sperm is interesting as these highly specialized cells have a small proteome (Ainsworth, 2005) and the expression of

unnecessary proteins would be expected to be too costly. The role of CYP6G1 in the degradation of juvenile hormone is proposed although initial experiments did not show any effect of juvenile hormone, these were not thorough enough to exclude the possibility that CYP6G1 is capable of juvenile hormone metabolism and this is important in the reproductive fitness of the fly.

Although the precise molecular mechanism whereby over-expression of CYP6G1 enzyme confers a maternally derived advantage remains to be elucidated, these results are important for the population genetics of insecticide resistance. They demonstrate that, contrary to conventional population genetic theory, insecticide resistance can show a benefit in the absence of selection, rather than a cost. The *Accord* LTR insertion allele of *Cyp6g1* has swept to a high frequency in *D. melanogaster* populations around the world (Catania, Kauer et al. 2004) indicating it confers an adaptive phenotype to its host. It is not clear exactly what has driven this sweep, although selection by insecticides is possible. Data indicate that the *Accord* insertion confers resistance to a broad spectrum of widely used chemical insecticides. The potential female linked reproductive advantage associated with the *Accord* allele presented here, may have some capacity to explain the selective sweep in addition to chemical selection. Further analysis of the fitness of *Cyp6g1* genotypes in the field is required. In addition work showing *Cyp6g1* over expression directly results in the fitness advantage described is necessary to eliminate the hypothesis that the fitness advantage resulting from closely linked genes.

The study shows that UK cat flea populations carry putative resistance associated mutations within *Rdl*. Further work is necessary to confirm the correlation between the presence of the resistance mutation and resistance to the compound fipronil. This work could include several approaches. The use of pressurisation to select for fipronil resistance within a population of fleas with an initially low resistant allele frequency would be an obvious approach. A subsequent increase in the resistance allele in such a population would be an indication that the mutation confers an increased resistance to the selective agent. The main problem with this technique is that fleas require a large population size to be maintained, however selecting with a high dose of a compound is likely to reduce the population size greatly. Using low doses of a

compound to select for resistance is likely to select for other minor forms of resistance. An alternative is to undertake more extensive sampling of treated and untreated cats is required to look for evidence of control failures in the presence of a high frequency of resistance-associated point mutations. The relative ease of the assay makes this a feasible approach however the costs associated with the assay are still high.

Research into the molecular basis of insecticide resistance provides a valuable insight into a fascinating and important biological phenomenon. Resistance research focuses not on the complete eradication of pest species, since this is likely to be unobtainable, instead research concentrates on the management of resistance and prolonging susceptibility in the field. The improvement of our understanding of resistance allows a better understanding the methods that insects employ to overcome susceptibility, enabling the design of improved, more specific insecticides. The implementation of rational insecticide design and a detailed analysis of insect detoxification systems we can develop the most effective compounds and prolong their effectiveness. The aims of this study were to address specific questions relating to two mechanisms of insecticide research. The results obtained help us understand the mechanisms and consequences of resistance, however they also present new questions that must be answered in order to further improve our control of resistance in pests.

Appendices

A1 STANDARD FLY DIET

1,300 ml H_2O
7.5 g plant agar
16 g yeast
80 g oats
138 g black treacle
17 ml acid mix (273 ml H_20, 206 ml propionic acid, 21 ml phosphoric acid)
20 ml 10% (w/v) tegosept (p-hydroxybenzoic acid) in ethanol

1. Add the agar to the 1000 ml water and microwave on full power for 3 min
2. Mix the oats, treacle and 300 ml water and microwave on full power for 30 s
3. Combine the two liquids and add the yeast
4. Microwave on full power for 10 min, stirring every 2 min
5. Add the acid mix and tegosept and pour into bottles

Taken from Ashburner and Wright, 1978

A2 EGG LAYING AGAR

9 g plant agar
300 ml H_2O
100 ml fruit juice concentrate
10 g sucrose
0.5 g tegosept

1. Boil the agar in the water
2. Boil the sucrose and tegosept in the fruit juice
3. Combine the liquids and pour

A3 RINGERS DISSECTION MEDIUM

130 mM NaCl

5 mM KCl

1.5 mM $MgCl_2$ ($6H_2O$)

A4 EGG WASH BUFFER

0.4 g NaCl

0.2 ml Triton X-100

1 l ddH_2O

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